105

THE QUANTITATIVE COMPLEMENT FIXATION TEST

WITH SPECIAL REFERENCE TO LOW TITRED SERA

by DORIETHY MOUNTFORD

(From the Department of Pathology, University of Melbourne).

(Accepted for publication 30th October, 1952.)

Wadsworth, Maltaner and Maltaner (1938) developed a technique with a quantitative basis, to investigate the complement (C′) fixation reaction with syphilitic serum.

In the qualitative C′ fixation reaction, the tests are interpreted by the varying degrees of haemolysis, which result from the activity of C′ remaining after fixation with immune serum and antigen. Thus the first step in the quantitative standardization of the reagents was a precise method for determining C′ activity. This was done by determining the amount of C′ required for 50 p.c. haemolysis of a standardized suspension of sensitized sheep cells. If the results of a C′ titration are plotted (volume of C′ against p.c. haemolysis) a sigmoid curve results. Consequently the 50 p.c. haemolysis titre is a more accurate measurement of complementary activity than complete or no haemolysis, since a given change in C′ in the 50 p.c. haemolysis region causes a relatively greater change in the degree of haemolysis. In this way the amount of C′ before and after fixation could be accurately titrated. The haemolysis curve can be described by the formula of von Krogh (1916), which was derived as an adsorption formula, and found to fit the haemolysis curve:

$$x = K\left(\frac{y}{1-y}\right)^{1/n} \qquad \text{where}$$

x = amount of C′; y = degree of haemolysis; K = constant, and is the 50 p.c. unit of C′; $1/n$ = constant and depends on the slope of the curve.

By the use of this equation conversion factors can be calculated for given conditions, so that the amount of C′ for 50 p.c. haemolysis can be calculated from results giving between 25–75 p.c. lysis, (that is, the amount of C′ for 50 p.c. lysis need not be determined directly).

In the test varying quantities of C′ were set up with the immune serum and the quantity of antigen required for a maximum reaction. In high titred sera a range of dilutions of serum was used. Varying quantities of C′ caused the value of $1/n$ to vary, and this necessitated individual sets of conversion factors to calculate the number of $C'H_{50}$ (the 50 p.c. unit of C′) for each quantity of C′ employed. This difficulty was overcome by Mayer *et al.* (1948)

by using a single quantity of C′ in large excess. A single set of conversion factors based on a mean value of $1/n = 0{\cdot}2$ sufficed for different immune systems and varying experimental conditions.

In the original quantitative method (Wadsworth *et al.*, 1938) the degrees of lysis were read by eye against colour standards. This technique was improved by reading the haemoglobins in a spectrophotometer (Mayer *et al.*, 1946 a; Kent *et al.*, 1946).

Also in the first method the quantities of C′ were varied so that when the haemolytic system was added to the fixation mixture, sufficient C′ was left to yield only partial lysis (approximately 1 $C'H_{50}$). This meant that the per cent. C′ fixed was high.

The quantitative test was further applied (Wallace *et al.*, 1950) in determining the titre of an immune serum. As stated these workers used a large excess of C′ and expressed the titre as the reciprocal of the volume of antiserum which fixes 50 p.c. of the available C′ (100 $C'H_{50}$) in the presence of the optimally reactive dilution of antigen. The 50 p.c. end titre was chosen due to the sigmoid nature of the fixation curve (see Fig. 2).

In this present work, the titres of a range of rabbit antisera against egg albumin have been determined using the method of Wallace *et al.* (1950). The same type of fixation curves (for varying antibody and antigen) were obtained.

The test employs very small quantities of antibody (0·5–4·0 μg. of antibody nitrogen) and has a precision of about 5 p.c. It might be thought, therefore, that the test would be applicable to the detection and determination of antibody in low titred sera, including a serum below the level at which visible precipitation reactions occur.

Osler and Heidelberger (1948 b) investigating cross reactions between pneumococcal type III and type VIII polysaccharides and corresponding antibodies, obtained marked anticomplementary effects when the dilution of immune sera (rabbit) used in the test was less than approximately 1 : 35. An attempt was made, therefore, to determine the percentage error in the fixation reaction due to a high concentration of serum, and the limiting dilution of serum beyond which the error would become negligible.

Materials and Methods.

Buffer solution. Veronal buffer was used as diluent for all reagents and reaction mixtures. Preparation: (Wallace *et al.*, 1950) 83·8 gm. NaCl, 2·52 gm. $NaHCO_3$, 3·00 gm. sodium 5–5 diethyl barbiturate, 4·60 gm. 5–5 diethyl barbituric acid, 1·0 gm. $MgCl_2.6H_2O$, 0·2 gm. $CaCl_2.2H_2O$. The acid was dissolved in 500 ml. of boiling glass-distilled water, the other reagents were dissolved separately, and the mixture made up to 2 litres with distilled water. This concentrated solution was stored at 4° C. and diluted 1: 5 as required. Mayer *et al.* (1948) found that 0·1 p.c. inactivated normal rabbit serum (N.R.S.), included in the diluent, reduced the adsorption of a protein antigen on the glass surfaces. Thus the quantity

of C′ apparently fixed was increased. The N.R.S. was later replaced with 0·1 p.c. gelatine (Wallace *et al.*, 1950). In this present work, tests (with a system where there was only one optimum dilution of antigen) showed that the addition of 0·1 p.c. gelatine had no effect on the amount of C′ fixed, and therefore it was not included in the diluent. The concentration of serum in the reaction mixture, due to the immune serum and/or the C′, was always greater than 0·1 p.c.

Glassware. All glassware was cleaned in a mixture of potassium dichromate and concentrated sulphuric acid and rinsed at least twelve times with tap water. It was found that insufficient washing caused irregularities in the tests, due to inactivation of the complement. Fixation tests were performed in tubes that had been rinsed (a) twelve times in distilled water, (b) twelve times in tap water and a final rinse in distilled water, (c) twelve times in tap water and a final soaking for six hours in distilled water. The values for $1/n$ and the volume of complement for 50 p.c. haemolysis were identical under all circumstances. All tests were carried out in test tubes 5 in. × ¾ in. 10, 5, 3, 2 and 1 ml. calibrated (for blowing) bulb pipettes and 5, 1·0 and 0·1 ml. calibrated graduated pipettes were used for measurements. In all cases the fluid was delivered by placing the tip of the pipette near the level of the liquid in the bottom of the tube.

Antigen. Crystalline ovalbumin (fowl) was prepared by the method of Kekwick and Cannan as given in Kabat and Mayer (1948). A stock solution of approximately 1 μg. $C_{ea}N$ per ml. was kept at 4° C. and dilutions prepared each day.

Antisera. Antisera were prepared by injecting rabbits subcutaneously into the foot pad with a Seitz-filtered solution of ovalbumin. Group 1a consisted of animals given a single injection of 10 mg. ovalbumin and bled at seventeen days. In group 1b were the same animals given, after twelve weeks, a further injection of 10 mg. and bled on the fifth, seventh and tenth days. The same animals (group 1c) were again given, after a further ten weeks, a third injection of 10 mg. ovalbumin plus an adjuvant and bled on the third, sixth and tenth days. A group 2a, other animals, were given a mixture of 10 mg. ovalbumin plus adjuvant and bled on the ninth and eighteenth days. Thus a range of sera with varying titres was obtained. Some sera were stored at —24° C. and heated to 56° C. for thirty minutes just before use. Other sera were Seitz-filtered and allowed to stand at room temperature until the complement had inactivated, and then kept at —24° C. The antibody titres were not affected by these variations in treatment (*vide infra*).

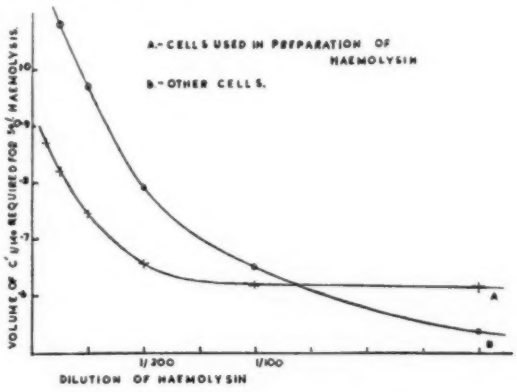


Fig. 1. Titration of haemolysin.

Haemolysin. Haemolysin was prepared by the injection of sheep red cells into a rabbit as described by Wadsworth (1947). The serum was heated at 56° C. for thirty minutes to inactivate the complement and stored in 0·5 ml. quantities at —24° C. The haemolysin was titrated as in Wadsworth (1947); that is, the volume of C′ required for 50 p.c. haemolysis was determined for varying dilutions of haemolysin. The optimum dilution is chosen as that

beyond which further increase fails to enhance appreciably the haemolytic activity of complement. The optimum dilution was determined for each new batch of red cells, and although a typical hyperbolic curve was obtained with the cells used for the preparation of the haemolysin, with cells from other animals the curve did not always flatten out (Fig. 1). However, the volume of C′ required for 50 p.c. haemolysis was approximately the same. The optimum dilution of haemolysin so determined, did not agglutinate the red cells.

Sheep erythrocytes. 250 ml. of sheep blood were collected aseptically into a sterile flask containing an equal volume of Alsever's solution (Bukantz *et al.*, 1946). The blood was stored at 4° C. After four days the fragility of the red cells remained constant for at least eight weeks (that is, the value of $1/n$ in the von Krogh equation remained constant and equal to $0{\cdot}2 \pm 10$ p.c.). Before use about 5 ml. of packed cells were washed at least three times with 40 ml. of buffer solution. The fragility of the washed packed red cells remained constant for two days at 4° C.

Complement. Pools of sera were prepared from 36 guinea-pigs. Since it was found that from 20 to 30 p.c. of the individual sera contained heterophil antibody to sheep red cells, the pooled complementary serum was absorbed with 5 ml. of packed sheep red cells per 100 ml. of guinea-pig serum. This was allowed to stand for twenty minutes at 4° C. with constant mixing, the red cells removed by centrifuging and the process repeated. However, this absorption with sheep cells was found to be unnecessary, if the serum from each individual guinea-pig's blood was pooled, mixed, and allowed to stand for twenty minutes at 4° C., before centrifuging. The complement was stored in ampoules of 5 ml. quantities at —24° C. The haemolysis titre and the fixation properties of the complement on storage did not deteriorate. The guinea-pig blood in the above preparation was not allowed to stand overnight and the above procedure was completed in one day. An ampoule once opened and allowed to thaw was discarded.

Absorptiometer. The degrees of haemolysis were measured in a Hilger "Spekker" absorptiometer, using a yellow green filter 605, with a maximum transmission at a wavelength of 5,800 Å.

Calibration. A 5 p.c. solution of red cells was prepared and a 1:40 dilution used for a cell count in a haemocytometer. From the mean of twenty counts the 5 p.c. solution was adjusted to contain 10^9 cells per ml. 1 ml. of this solution was then added to various amounts of 0·1 p.c. Na_2CO_3 (0·1 p.c. Na_2CO_3 yields an optically clear lysate) to correspond to a range of haemolysis between 20 and 80 p.c. and the readings taken on the absorptiometer using $1{\cdot}0 \times 0{\cdot}3$ cm. cuvettes and against distilled water as a blank. The calibration curve so obtained was a straight line between 25 and 75 p.c. haemolysis. The sensitized cell suspension was standardized each day so that 1 ml. of cells added to 11 ml. of 0.1 p.c. Na_2CO_3 solution gave a reading of $0{\cdot}435 \pm 0{\cdot}003$. This corresponded to 50 p.c. haemolysis in the haemolysis tests (*vide infra*). A standard solution of cobaltous chloride was prepared in distilled water to give a reading of 0·435 and the instrument checked each day; the reading was always constant. Cuvettes were rinsed with each test solution before being filled.

EXPERIMENTAL.

The complement fixation tests.

The C′ was diluted to contain 10 $C'H_{50}$ units/ml. in the final reaction mixture after eighteen hours at 0–4° C. This necessitated a dilution of guinea-pig serum approximately 1:8. One millilitre of diluted C′ was added to 0·5 ml. antibody dilution, mixed, and then 0·5 ml. antigen added within ten minutes. A control of every antibody dilution plus C′ and buffer was

included. The antigen was not anticomplementary. In cases where only a small quantity of immune serum was available a total of 0·8 ml. was found satisfactory. The tests were left at 0–4° C. for eighteen hours and then 10 ml. of ice cold buffer added to each tube. The C′ dilutions did not deteriorate in the following two hours, provided they were kept cold. A 5 p.c. solution of cells was prepared, an equal volume of an optimal dilution of haemolysin added, and the suspension standardized. The mixture was incubated at 37° C. for ten minutes and allowed to stand for twenty minutes at room temperature before use. Haemolysis tests were set up using 1 ml. of red cell suspension, plus C′ dilution, and the volume made up to 6 ml. with buffer. Two volumes of C′ were used for each determination, to give haemolysis between 25 and 75 p.c. The tubes were incubated for forty-five minutes at 37° C. and shaken every fifteen minutes. The mixtures were transferred to centrifuge tubes and spun at 2,000 revolutions per minute for fifteen minutes. The concentrations of haemoglobin were read in the absorptiometer against a spun blank of unsensitized cells and buffer and a dilution of C′. This generally lowered the reading by approximately 0·015 as compared with distilled water. From the degree of haemolysis the number of $C'H_{50}$ left in the mixture was calculated by the use of the conversion factors (Mayer *et al.*, 1946 b) derived from the von Krogh equation of $x = K\left(\frac{y}{1-y}\right)^{1/n}$ with a value $1/n = 0{\cdot}2$. The number of $C'H_{50}$ fixed was obtained by subtracting the residual C′ from the number of $C'H_{50}$ in the control, and then expressed as the p.c. C′ fixed. A protocol of a typical determination is given in Table 1. In some of the experiments $1/n$ was found to be greater than 0·2 due to the presence of a high concentration of immune serum (1, 1 : 2, in Table 5). In these cases three determinations were made in the range of partial haemolysis and the conversion factors calculated by plotting log x against log $\frac{y}{1-y}$ (Wadsworth *et al.*, 1947). In this way, the titres of various sera were obtained, and the fixation curves plotted. Some representative curves are shown in Figs. 2 and 3. These are plotted from the data given in Table 2. Curves 9, 10 and 11 are plotted on a much smaller antibody scale in Fig. 3, but portion of curve 9 is also shown on Fig. 2. The spacing of the serum dilutions depended on the titre of the serum. This can be seen by the various slopes of the curves but, in general, closer dilutions than serial doubling dilutions are required. When the approximate dilution of a serum is known (as in repeated bleedings), 3–4 dilutions of a serum were found satisfactory; in other cases a wide range of dilutions may be necessary. This author found that some idea of the titre could be gained by the time, quantity and zone of precipitation in a capillary tube, with a standard dilution of antigen. It was also found essential to use three serial doubling dilutions of antigen to determine the optimal fixation.

The optimum dilution of antigen for each individual serum also varied with the per cent. fixation. Three fixation curves with varying amounts of antigen are shown in Fig. 4 (Table 3). In routine determinations the optimum dilu tion of antigen was chosen by eye (the tube showing least haemolysis, for the same volume of C′ dilution) and then read in the absorptiometer.

TABLE 1.

Protocol for the determination of the titre of an immune serum.

Dilution of immune sera, added to test	Volume of C′ (reaction mixture diluted 1/6)	Absorptiometer reading	P.c. haemolysis	Conversion factors for $1/n = \cdot 2$	No. of C′H_{50} units/ml. left after fixation	No. of C′H_{50} units/ml. fixed	P.c. fixation	Dilution of serum giving 50 p.c. fixation
					·98/2 × 6			
1/80	2 ml.	·412	46·3	·98	29·4	6·98	70	
control	·6 ml.	·490	47·8	·992	9·92			
1/100	1·6 ml.	·467	54·2	1·044	3·91	6·29	62	1/133
control	·6 ml.	·442	50·9	1·018	10·2			
1/140	1·3 ml.	·530	63·3	1·13	5·21	4·71	47·5	
control	·6 ml.	·490	47·8	·992	9·92			

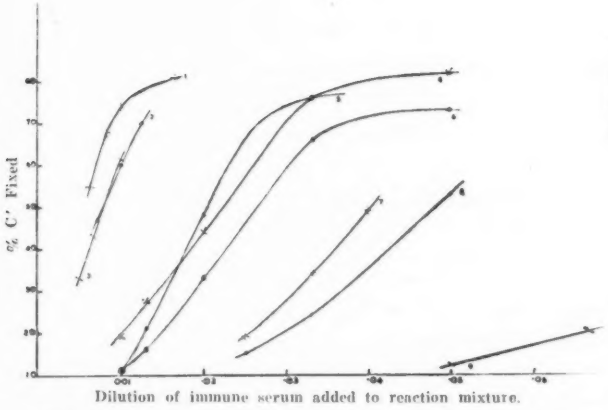


Fig. 2. Maximum fixation of C′, with varying dilutions of immune serum and optimum antigen.

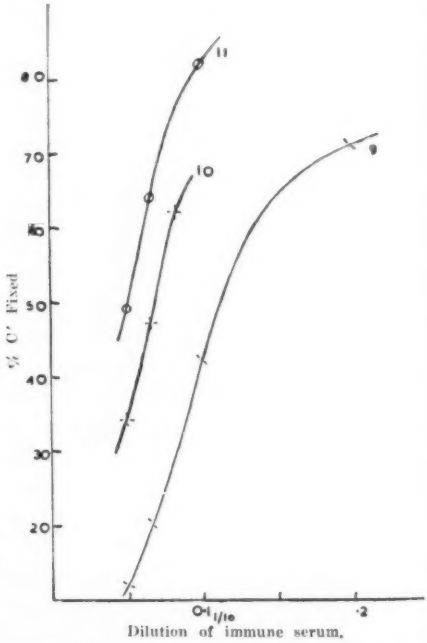


Fig. 3. Maximum fixation of C′ with varying dilutions of immune serum and optimum antigen.

TABLE 2.

Maximum fixation of C', with varying dilutions of immune serum and optimum antigen.

	Per cent. C' fixed.										
Serum	42	53	22	22	50	100	60	31	31	129	50
Curve No. Fig. 2 and 3	1	2	3	4	5	6	7	8	9	10	11
Group No.	1C	1C	1C	1B	2	1C	2	1B	1C	2	2
Day of bleeding	6	10	6	7	9	10	18	10	3	9	18
Dilution of immune serum											
1: 5									71		
1: 10									42		82
1: 12										62	
1: 15					85				20	47	64
1: 20				82		73		53	12	34	49
1: 25							49				
1: 30				76	76	66	34	24			
1: 40							19	15			
1: 50				44	48	33					
1: 60	81										
1: 75				27·5	21	16					
1: 80		70									
1: 100	74	60	61·5	19	11	11					
1: 120	68										
1: 130				0	0	0					
1: 140		47									
1: 150			43·5								
1: 160	55										
1: 200			33								

TABLE 3.

Maximum fixation of C' with varying antigen and constant antibody.

		Per cent. C' fixed.		
	Serum	22	50	100
	Curve Fig. 4	4	5	6
	Group	1B	2	1C
Dilution of antigen	Day of bleeding	7	9	10
1: 2		4	30	8
1: 4		16	38	39
1: 8		45	36	38
1: 16		52	27	38
1: 32		39	20	36

The effect of normal rabbit serum on the fixation reaction.

It was found that when a high concentration of serum was used, a large proportion of the C′ was fixed in the serum control. This is illustrated in Table 4. These sera were heated at 56° C. for thirty minutes, and in addition the first four sera were diluted 1:2 and heated at 62° C. for fifteen minutes. However, the percentages C′ fixed in the fixation tubes and the controls were the same.

TABLE 4.

The anti-complementary effect of immune serum in low dilution.

Serum	Group	Day of bleeding	Serum titre	Dilution of serum added to test	P.c. C′ fixed in controls
42	1C	3	2·4	1:3	42
42	1C	3	2·4	1:4	13
42	1B	10	5·6	1:6	20
42	1B	10	5·6	1:8	13
42	1B	10	5·6	1:10	8
31	1C	3	3·2	1:10	10
42	1B	7	7·2	1:10	8
100	1B	7	10·8	1:10	—
100	1B	7	10·8	1:15	—
22	1B	10	11·2	1:15	7
42	1B	7	7·2	1:15	6
31	1C	3	3·2	1:15	—
42	1B	10	5·6	1:20	3
42	1B	7	7·2	1:20	—
31	1B	10	8·4	1:20	—
100	1B	10	6·8	1:20	—
48	1C	3	17·6	1:30	4
34	1C	14	15·2	1:30	—
22	1C	3	12·8	1:30	—
31	1C	14	12	1:30	8
31	1B	7	14·8	1:30	3
48	1C	3	17·6	1:40	—
34	1C	10	25·2	1:40	—
22	1B	10	11·2	1:40	—
22	1B	7	20·4	1:40	—
48	1C	3	17·6	1:60	—
34	1C	14	15·2	1:60	—
42	1C	14	42·4	1:60	—
22	1C	3	12·8	1:60	—
53	1C	14	53·2	1:80	—
34	1C	10	25·2	1:90	—
22	1C	10	52	1:100	—
31	1C	14	18·8	1:100	—
22	1B	7	20·4	1:200	—

It was also noticed that the proportion of C′ fixed increased with a decrease in the quantity of serum up to a certain value, and then decreased in the normal way. Therefore, it was decided to see whether the presence of normal rabbit serum (N.R.S.) interfered with the fixation reaction. For this purpose an immune serum with a very high titre was chosen (requiring a dilu-

tion of 1 : 140 and thus eliminating the effect due to serum), set up with the usual range of antigen dilutions, the same quantity of C′, but in half the volume, and with the addition of one volume of N.R.S. so that the total volume was the same as before. Various dilutions of N.R.S. were used. The N.R.S. was treated in two ways. One portion was Seitz-filtered and allowed to stand at room temperature until the C′ had deteriorated. After two weeks the C′ could not be detected in the dilution normally present in the haemolysis test. The other portion of each N.R.S. was heated at 56° C. for thirty minutes just prior to use. The reagents for the test were added in the following manner: 1 ml. immune serum, 1 ml. N.R.S., 1 ml. of C′ (40 $C'H_{50}$) and 1 ml. of antigen. Buffer replaced the antigen in the controls. A control test was included with no N.R.S. to check the activity of the immune system each day. This is shown in column A in Table 5. The reagents were added within ten minutes, incubated at 0–4° C., and the residual C′ titrated as before. The number of $C'H_{50}$ remaining in the total reaction mixture after fixation is shown in Table 5. The number of $C'H_{50}$ remaining in the controls (no antigen) is also shown in row 3, together with the calculated proportion of C′ fixed.

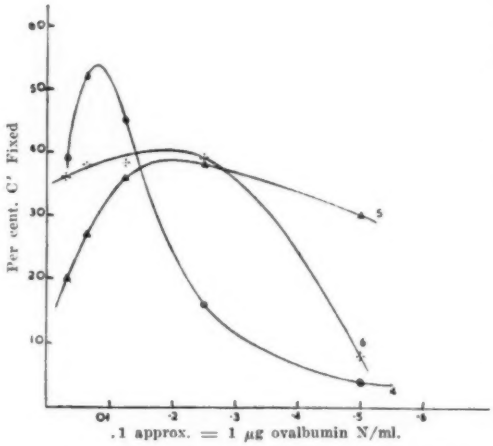


Fig. 4. Maximum fixation of C′ with varying antigen and constant antibody.

The one dilution of antigen was optimum throughout. Except in the case of Seitz-filtered serum D the same number of $C'H_{50}$ was added to each test.

The effect of heat on the antibody content of sera was investigated, by determining the per cent. fixation of Seitz-filtered and heated immune sera of varying titres, for the same dilution. The results are shown in Table 6.

Results.

In the normal C′ fixation tests (Table 4) it can be seen that an appreciable amount of C′ is inactivated by the immune serum alone in dilutions up to 1 : 10. Beyond a dilution of 1 : 20 the anticomplementary effect is small (due to inactivation of C′ in the controls). There appears to be no relation between the day of bleeding and the anticomplementary effect as was found by Donnelly (1951) using a virus (influenza) system; but rather the anticomplementary effect appears to depend on the concentration of serum. For example, serum 42, 1C

TABLE 5.

The effect of normal rabbit serum on the fixation reaction.

		Control test A	B	C	N.R.S. Seitzed W					Control test	N.R.S. Seitzed Y						
Dilution of N.R.S. added to test	1	—	1	1:2	1:4	1:8	1:16	1:32	1:64	—	1	1:2	1:4	1:8	1:16	1:32	1:64
Number of $C'H_{50}$ units left in test after fixation	2	15.8	36.8	20	15.7	15.7	14.8	14.6	14.2	15.4	33.7	24.9	18.1	17.9	18	18	16.6
Number of $C'H_{50}$ units in control (no antigen)	3	45	38.3	21.8	25.3	26.7	33.4	41.4	42.4	40	31.6	27	28.5	29.7	37.4	37.8	36.6
P.c. C′ fixed in reaction	4	65	4	8	38	41	56	65	67	62	—	8	37	40	52	52	55
P.c. error in fixation	5	—	94	88	41	37	14	—	—	—	100	87	40	36	16	16	11
Difference in number of $C'H_{50}$ in fixation (B—A) (C—A) etc., column 2	6	—	21	42	—	—	—	—	—	—	18	10	3	3	3	3	1
Difference in number of $C'H_{50}$ in controls (A—B) etc., column 3	7	—	4	20	17	15	9	—	—	—	8	13	12	10	3	2	3
P.c. error with A as control	8	—	82	20	4	4	—	—	—	—	75	44	11	11	11	11	5
			N.R.S.		W		56° 30′				N.R.S.		Y		56° 30′		
Number of $C'H_{50}$ units left in test after fixation	2	15.8	36	26.1	22.4	21.4	18.6	16.4	15.2	15.8	35.5	22	18.5	16.1	14.6	14.4	14.2
Number of $C'H_{50}$ units in control (no antigen)	3	38.4	36.8	33.8	35.2	32.1	36.1	37.8	36.4	38.4	45.5	34.4	32.7	30.4	34.2	36.0	35.9
P.c. C′ fixed in reaction	4	59	—	23	37	33	49	57	58	59	20	36	44	47	57	60	60
P.c. error in fixation	5	—	100	61	38	43	18	—	—	—	66	39	76	20	—	—	—
Difference in number of $C'H_{50}$ in fixation (B—A) (C—A) etc., column 2	6	—	20	10	7	6	3	—	—	—	21	8	4	2	—	—	—
Difference in number of $C'H_{50}$ in controls (A—B) etc., column 3	7	—	—	3	2	5	—	—	—	—	+10	2	3	6	—	—	—
P.c. error with A as control	8	—	96	50	33	29	15	—	—	—	100	34	18	5	—	—	—

TABLE 5 (continued).

The effect of normal rabbit serum on the fixation reaction.

		Control test A	B	C	N.R.S. Seitzed D				Control test		N.R.S. Seitzed X					
Dilution of N.R.S. added to test	1	—	1	1:2	1:4	1:8	1:16	1:32	1:64	—	1	1:2	1:4	1:8	1:16	1:32	1:64
Number of $C'H_{50}$ units left in test after fixation	2	15.8	38.9	47.9	39.8	24.9	21.3	19.4		15.2	15.8	18.3	10.9	13.6	13.2	14.6	
Number of $C'H_{50}$ units in control (no antigen)	3	41.7	41.2	53.6	53.1	49.9	43.5	43		32.7	23.1	23.3	22.9	25.3	24.5	30.3	
P.c. C′ fixed in reaction	4	62	18	11	25	50	50	55		53.5	33	21.5	52	46.5	46	52	
P.c. error in fixation	5	—	68	82	60	19	19	11		—	40	60	3	13	14	—	
Difference in number of $C'H_{50}$ in fixation (B—A) (C—A) etc., column 2	6	—			a different					—	—	3	—4	—2	—2	—	
Difference in number of $C'H_{50}$ in controls (A—B) etc., column 3	7	—			dilution of C′ added to each					—	10	9	10	7	8	—	
P.c. error with A as control	8	—			test					—	7	21	28	12	9	—	

		N.R.S.	D			56° 30′			N.R.S.	X			56° 30′	
Number of $C'H_{50}$ units left in test after fixation	2	15.4	27	24.9	23.1	22.3	18	16.9	15.4	35.2	19.6	19.2	16	16.4	14.4	14.7
Number of $C'H_{50}$ units in control (no antigen)	3	38.4	27	38.4	32.1	39	36	46.2	40	47.4	35.8	30.6	30.3	37.1	36.9	37.1
P.c. C′ fixed in reaction	4	60	—	12	28	43	51	63	62	21	45	37	47	56	61	61
P.c. error in fixation	5	—	100	80	53	28	15	—	—	66	28	40	24	10	—	—
Difference in number of $C'H_{50}$ in fixation (B—A) (C—A) etc., column 2	6	—	12	10	8	7	3	—	—	20	5	4	1	1	—	—
Difference in number of $C'H_{50}$ in controls (A—B) etc., column 3	7	—	—	10	6	—	2	—	—	+10	1	6	7	—	—	—
P.c. error with A as control	8	—	51	41	34	30	11	—	—	20	23	23	7	8	—	—

on the third day after an injection, with a serum dilution of 1:3 fixed 42 p.c. C′, but serum 22, 1C also on the third day with a dilution of 1:30 did not fix any C′ in the control.

In the experiments in which N.R.S. was included (Table 5), the per cent. error in fixation (calculated from row 2) is shown. Rows 6 and 7 were calculated in order to see where the errors were occurring (that is, in the fixation reaction, or controls or both).

Row 6 is the difference in the amount of C′ left in the fixation mixture when N.R.S. was present, and row 7 the difference when N.R.S. was present in the control.

The presence of N.R.S. introduced a large error in the amount of C′ fixed, in all cases up to a dilution of at least 1:16 (Table 5, row 5). Also the results differ for each N.R.S.; for example, although the same amount of antibody was present in each case, the per cent. fixation for a N.R.S. dilution of 1:8 varied from 33–50.

The large errors obtained are not clearly due to any one factor. For example, in Seitz-filtered serum W there was no difference in the residual C′ in the fixation test beyond a dilution of 1:4, but there was a vast difference in the amount of C′ in the controls. However, in heated serum D there was a difference in the residual C′ in the fixation test, as well as the controls.

Although heating destroys some of the anticomplementary power of the N.R.S., a large error persisted up to a dilution of 1:16. Nevertheless, in Seitz-filtered sera D and Y there was still an error with a dilution of 1:32, which appears to have been removed on heating the sera. In all cases the error appears to be reduced (rows 5 and 8) if the calculations are made with the control containing immune serum and C′ alone (that is, C′ alone).

Heating the immune serum (Table 6) does not appear to have any effect on the complement fixing power of the anti-ovalbumin antibody (cf. Bawden and Kleczkowski, 1942). The difference in fixation between the Seitz-filtered and heated serum for the first two sera in Table 6 can probably be accounted for by the high concentration of serum present.

TABLE 6.

The effect of heating immune serum.

Serum	Group	Day of bleeding	Dilution of immune serum	P.c. fixation with Seitzed sera	P.c. fixation
34	1B	7	1:3	53	61
42	1B	5	1:5	39	50
31	1B	5	1:10	64	63
31	1B	5	1:25	67	63
22	1B	5	1:30	62	64
22	1C	14	1:70	61	63
53	1C	14	1:90	60	64

DISCUSSION.

The technique for the quantitative determination of the titre of the C′ fixing power of an immune serum as described by Wallace *et al.* (1950) has been successfully applied to high titred sera, with a few minor alterations in technique (for example, smaller volumes were used for convenience).

The fixation curves (Figs. 2 and 3) obtained by plotting varying quantities of antibody and the per cent. C′ fixed, have an S-shape. The slope of the S-shaped curve depended on the titre of the serum (that is, dilution of serum used), as the curves using high dilutions of serum were much steeper. The slope of the curve did not necessarily depend on whether it was a response to a primary, secondary or tertiary injection. For example, curves 10 and 11 (Fig. 3) are primary responses whereas curve 9 is a tertiary response. Similarly with curves 5 and 6. Thus the slope of the curve within this range of titres may depend on the presence of serum.

Wallace *et al.* (1950) found that when the amounts of antibody N (rabbit anti-bovine-serum-albumin antibody) were plotted against the number of $C'H_{50}$ fixed, S-shaped curves resulted. The slope of the S-shaped curve of the first course anti-sera (produced after a series of fourteen injections of a total of 44 mg. of bovine serum albumin) was much less than the second course anti-sera (after a further 60 mg. of bovine serum albumin). These sera were, in general, of a much higher titre than those used in the present work, although several (137-1 and pool B) were of the same order. However, the slope of the S-shape curves bore no relation to the volume of serum used. This difference may be due to the prolonged period of immunization used as compared with the single injection in the present work.

Although it was found necessary to ascertain the optimum dilution of antigen in each case, the effects of varying amounts of antigen have not been studied any further.

Wallace *et al.* (1950) have defined the end titre as the reciprocal of the volume of anti-serum, which fixes 50 out of 100 $C'H_{50}$ employed in the test, in the presence of the optimally reactive dilution of antigen. They have also introduced the term $C'F_{50}$, this being the reciprocal of the volume of anti-serum in ml. which fixes 50 p.c. of the available C′. It was not clear to this author whether "available C′" was meant to be 100 $C'H_{50}$ (10 $C'H_{50}$ in the final reaction mixture) or to any number of $C'H_{50}$. The value of $C'F_{50}$ would depend on the available C′ (Mayer *et al.*, 1948).

Where it is desirable to inactivate C′, heating would seem to be a satisfactory method, at least for this system, as the C′ fixation titre of the serum is not diminished. Heating also reduces the anticomplementary properties of the lower titred sera, and thus reduces the error in the per cent. fixation.

The results of other workers (Bawden and Kleczkowski, 1942; Casals and Palacois, 1941) suggest that heating at a higher temperature (60–65° C.)

might further reduce the anticomplementary effect, without diminishing the antibody content. For instance, Casals and Palacois (1941) using various virus systems, found that heating rabbit serum (diluted 1:2) at 65° C. for twenty minutes removed non-specific reactions and anticomplementary effects which were present in a dilution of 1:4 and 1:6. The antibody content was not diminished.

In this system heating the immune serum at 62° C. for fifteen minutes did not reduce the anticomplementary properties of the serum any more than heating at 56° C. for thirty minutes.

The presence of normal rabbit serum interferes with the fixation reaction, and this is not entirely due to the anticomplementary effect of the N.R.S. in the serum control. If one can assume the immune serum would have the same inhibitory effect on fixation as N.R.S., the quantitative C′ fixation test would then be unreliable at least with dilutions to 1:30. However, the error varies with individual sera, and in the normal tests (Table 4) the anticomplementary effects were small above a dilution of 1:10. It is interesting to note that Osler and Heidelberger (1948) found marked anticomplementary effects with rabbit serum at an apparent dilution of at least 1:35. Anticomplementary effects due to inactivation of the C′ in the serum controls were not encountered at this dilution in the present work (Table 4).

For these reasons this test cannot be considered as strictly quantitative when a high concentration of serum is present. However, further work in this laboratory (unpublished) has shown that differences in antibody content in successive bleedings (after one injection) from the same rabbit can be detected readily with serum dilutions as low as 1:2.

Wallace *et al.* (1950) do not regard the C′ fixation titre of an anti-serum as a direct measure of its "antibody content," since the quantity of antibody N (determined by quantitative precipitation methods) required for 50 p.c. fixation varied with the length of immunization. They found that the amount of C′ fixed by a given amount of antibody N after a first course of immunization was lower than that for the same amount of antibody N from second course antisera. Heidelberger and Kendall (1935) using crystalline ovalbumin and rabbit anti-ovalbumin serum performed a series of quantitative precipitation tests on three samples of serum from one rabbit at different stages in a long course of immunization. They found under standard conditions that the ratio of antibody N to antigen N increased after each course of immunization. They were best able to explain this by suggesting that the antibody formed in the later stages of immunization is reactive with a larger number of chemically distinct groupings on the ovalbumin molecule. Further evidence supporting this is the broadening of the scope of cross reactions (Heidelberger and Kendall, 1934) and of the equivalence zone (Heidelberger and Kendall, 1935) on continued immunization.

The results of both these workers (Wallace *et al.*, 1950; Heidelberger and

Kendall, 1935) are consistent with the hypothesis that the C′ fixing titre of a serum depends on the number of such postulated reactive groupings between the antigen and antibody, and that the antibody N content of a serum depends on the number of groupings and the molecular weight of the antibody. Thus the C′ fixing power of an immune serum may be a truer measure of the "antibody content" in the sense of the number of reactive groupings, but this could best be decided by comparing C′ fixation titres, antibody N content and sensitive protective capacity tests (in animals) on the one serum.

SUMMARY.

The technique of Wallace *et al.* (1950) for the quantitative determination of the C′ fixing power of an immune serum has been successfully applied to high titred serum.

With low titred serum the test is no longer quantitative due to the large error produced by the serum.

An attempt was made to analyse the errors due to a high concentration of serum, but these were not found to be due to any one factor.

Heating to 56° C. for thirty minutes reduced the anticomplementary effect of the serum, without altering the antibody content. Heating to a higher temperature (62° C., for fifteen minutes) produced the same results.

The slope of the complement fixation curves appeared to be related to the concentration of antibody in the sample of serum, irrespective of whether the antibody had been produced in response to the first, second or third injection.

REFERENCES.

Bawden, F. C. and Kleczkowski, A. (1942): Brit. J. exp. Path., 23, p. 178.
Bukantz, S. C., Rein, C. R. and Kent, J. F. (1946): J. lab. clin. Med., 31, p. 394.
Casals, M. D. and Palacois, R. (1941): J. exp. Med., 74, p. 409.
Donnelly, M. (1951): Austral. J. exp. Biol., 29, p. 137.
Heidelberger, M. and Kendall, F. E. (1934): J. exp. Med., 59, p. 519.
Heidelberger, M. and Kendall, F. E. (1935): *Ibid.*, 62, p. 697.
Kabat, E. A. and Mayer, M. M. (1948): "Experimental Immunochemistry", Charles C. Thomas, Springfield, Ill.
Kekwick, R. A. and Cannan, R. K. (1936): Biochem. J., 30, p. 227.
Kent, J. F. (1946): J. lab. clin. Med., 31, p. 1,270.
Mayer, M. M., Eaton, B. B. and Heidelberger, M. (1946 a): J. Immunol., 53, p. 31.
Mayer, M. M., Osler, A. G., Bier, O. G. and Heidelberger, M. (1946 b): J. exp. Med., 84, p. 535.
Mayer, M. M., Osler, A. G., Bier, O. G. and Heidelberger, M. (1948): J. Immunol., 59, p. 195.
Osler, A. G., Mayer, M. M. and Heidelberger, M. (1948 a): *Ibid.*, 60, p. 205.
Osler, A. G. and Heidelberger, M. (1948 b): *Ibid.*, 60, p. 317.
von Krogh, M. (1916): J. infect. Dis., 19, p. 452.
Wadsworth, A. (1947): "Standard Methods", 3rd Edition. Williams and Wilkins, Baltimore, p. 397.
Wadsworth, A., Maltaner, F. and Maltaner, E. (1938): J. Immunol., 35, p. 217, and earlier papers.
Wallace, A. L., Osler, A. G. and Mayer, M. M. (1950): J. Immunol., 65, p. 661.

A COMPARISON OF THE MORPHOLOGY OF VACCINIA AND MYXOMA VIRUSES

by J. L. FARRANT AND FRANK FENNER

(From the Chemical Physics Section, Division of Industrial Chemistry, Commonwealth Scientific and Industrial Research Organization, Melbourne, and the Department of Microbiology, the John Curtin School of Medical Research, the Australian National University, Canberra).

(Accepted for publication 21st November, 1952.)

There is general agreement among virologists that, from the viewpoint of taxonomy, morphology is the most important single property of the animal-pathogenic viruses.

Study of stained smears of the elementary bodies of the virus of infectious myxomatosis with the light microscope (Rivers and Ward, 1937) established that in appearance they resembled the elementary bodies of vaccinia. This similarity was confirmed by electron microscopic examination of these viruses by Ruska and Kausche (1943), who showed that the elementary bodies of both were almost identical in size and exhibited the brick-shaped appearance characteristic of the pox viruses. Despite this evidence myxoma is not generally classified as a pox virus but has usually been grouped with Rous sarcoma and Shope papilloma viruses as a tumour-producing agent (e.g., Holmes, 1948). Detailed studies by one of us (F.F.) of myxomatosis in European rabbits revealed that there was a closer similarity between the pathogenesis of myxomatosis and the pox diseases than had previously been recognized. These considerations prompted us to undertake a more detailed electron microscopic examination of myxoma virus.

MATERIALS AND METHODS.

The materials used were the South American strain of myxoma virus (Fenner, Day and Woodroofe, 1952) and the "Mill Hill No. E.B. 102" strain of vaccinia virus investigated by Dawson and McFarlane (1948).

Elementary body suspensions of both viruses were prepared by scarification of the backs of rabbits. Vaccinia was reaped three days (Smadel and Hoagland, 1942) and myxoma five days after the scarification (Rivers and Ward, 1937). After spinning for five minutes at about 1,500 *g* in a horizontal centrifuge to remove cell debris, the virus particles in the supernate were washed three times by re-suspension in dilute buffer (0·004 M citric acid-disodium phosphate pH 7·0–7·2) after each cycle of centrifugation at 2,500 *g* for sixty minutes in a Spinco preparative ultracentrifuge. The elementary bodies were treated with 1·0 M NaCl for three days at 4° C. (Dawson and McFarlane, 1948) and washed several times in the Spinco centrifuge.

Specimens for electron microscopy were prepared by placing droplets of the virus suspensions on collodion supporting films, adding an equal volume of 1 p.c. osmium tetroxide solution and allowing the mixture to evaporate almost to dryness. The specimens were then washed with distilled water and when dry were shadow-cast with uranium.

RESULTS.

Fig. 1 shows that the elementary bodies of myxoma have the brick-shaped outline characteristic of viruses of the pox group; in addition they possess a central area of greater electron-scattering power similar to that demonstrated in vaccinia virus by Green, Anderson and Smadel (1942) and by Sharp, Taylor, Hook and Beard (1946) and in fowl-pox virus by Bang, Levy and Gey (1951). The profiles of the shadows indicate that the central body stands higher than the peripheral regions, and this was confirmed on occasions when rupture of the supporting membrane allowed the particles to be seen on edge. The close resemblance between myxoma and vaccinia viruses can be seen from Figs. 1 and 2.

Dawson and McFarlane (1948) demonstrated that the central body of vaccinia resisted peptic digestion whereas the outer part of the elementary body dissolved under this treatment. Similar observations have been made on fowl-pox virus (Bang, Levy and Gey, 1951). These findings led us to investigate the effect of this enzyme on myxoma virus. Elementary body suspensions were treated with a solution of purified pepsin at pH 3 for 20 minutes at 37° C. and electron micrographs of the preparation were taken after fixation with osmium tetroxide and shadowing with uranium. Fig. 3 illustrates that this treatment removed all the material lying between the central body and the peripheral membrane, both of which resisted the enzyme. The effect is identical with that which Dawson and McFarlane (1948) produced by the same treatment of vaccinia, a result since confirmed by ourselves and by Peters and Nasemann (1952).

Our determinations of the mean virus dimensions and their standard deviations are listed in Table 1.

TABLE 1.

	No. of particles measured	Mean length (mμ)	Mean width (mμ)	Mean thickness (mμ)
Myxoma virus	24	286 ± 15	230 ± 20	75 ± 10
Vaccinia virus	25	305 ± 15	227 ± 20	80 ± 10

They are dependent on magnification calibration of the electron microscope obtained by means of interferometrically-measured glass fibres (Farrant and Hodge, 1948). The small differences in the mean lengths and thicknesses

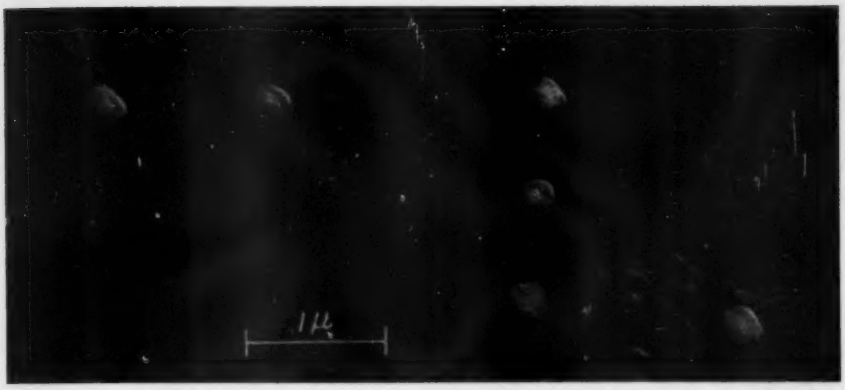


Fig. 1. Electron micrograph of myxoma virus fixed with osmium tetroxide and shadowed with uranium; ratio of shadow length to particle height, 4 to 1.

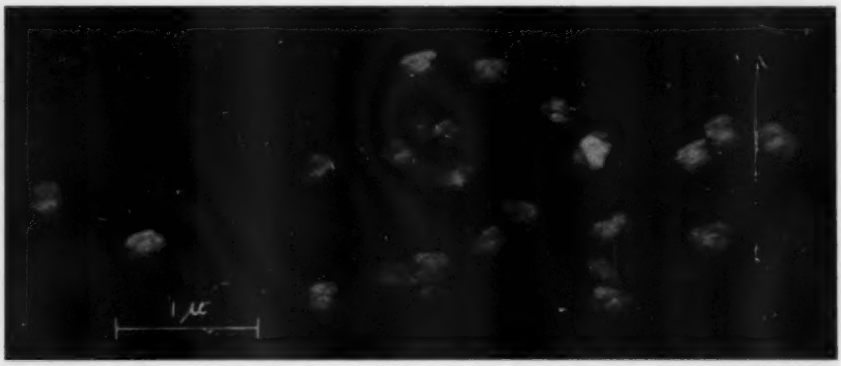


Fig. 2. Electron micrograph of vaccinia virus fixed with osmium tetroxide and shadowed with uranium; ratio of shadow length to particle height, 6 to 1.

of the elementary bodies of myxoma and vaccinia shown in Table 1 are not regarded as significant. The lateral dimensions of myxoma agree remarkably well with the values 287±21 by 233±19 mμ given by Ruska and Kausche (1943), and our values for vaccinia are not greatly different from the dimensions 262±28 by 209±24 mμ determined by these authors. Ruska and Kausche's observations, however, refer to non-fixed specimens. Our values differ somewhat from the dimensions 200 by 160 by 90 mμ found by Dawson and McFarlane (1948) for the same strain of vaccinia prepared in a similar fashion, fixed with osmium tetroxide and dried at room temperature. We cannot satisfactorily explain this difference.

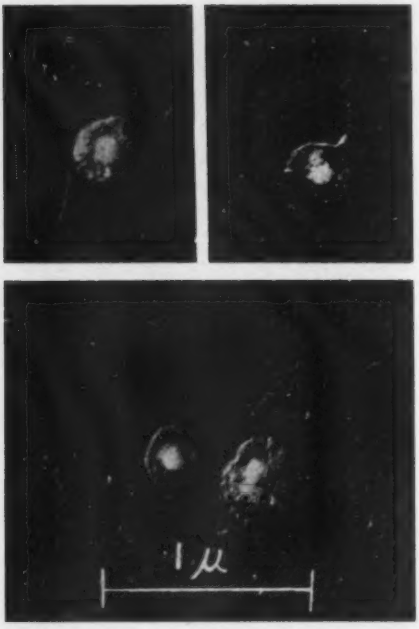


Fig. 3. Electron micrograph of myxoma virus digested with pepsin, fixed with osmium tetroxide and shadowed with uranium; ratio of shadow length to particle height 4 to 1.

Our investigations thus confirm the impression of the superficial morphological resemblances between the elementary bodies of myxoma virus and vaccinia virus given by previous studies with the electron microscope and the light microscope. Further, they demonstrate the existence of a similarity in the internal architecture of the elementary bodies of these two viruses. It is noteworthy that the existence of a central body resistant to peptic digestion has been demonstrated previously only in vaccinia and fowl-pox, two viruses with undisputed claim to membership of the pox group.

SUMMARY.

The elementary bodies of myxoma and vaccinia viruses are found to be identical in the electron microscope. Both have the brick-shape characteristic of the pox viruses. The dimensions of the osmium-fixed particles are $286\pm15 \times 230\pm20 \times 75\pm10$ mμ and $305\pm15 \times 227\pm20 \times 80\pm10$ mμ for myxoma and vaccinia respectively. Myxoma particles contain dense pepsin-resistant central bodies similar to those demonstrated in vaccinia and fowl-pox viruses by other workers. These facts, together with the recent demonstration of a close similarity between the pathogenesis of myxomatosis and the pox diseases, indicate that myxoma belongs to the pox group.

Acknowledgments. We are indebted to Dr. A. S. McFarlane for the strain of vaccinia virus used in these studies, and to Mr. G. L. Ada for carrying out centrifugation on the Spinco preparative ultracentrifuge.

We are grateful to Sir MacFarlane Burnet, Director of the Walter and Eliza Hall Institute, for making the facilities of the Institute available to one of us (F.F.) pending the completion of the laboratories of the University in Canberra.

REFERENCES.

Bang, F. B., Levy, E. and Gey, G. O. (1951): J. Immunol., 66, p. 329.
Dawson, I. M. and McFarlane, A. S. (1948): Nature, 161, p. 464.
Farrant, J. L. and Hodge, A. J. (1948): J. appl. Phys., 19, p. 840.
Fenner, F., Day, M. F. and Woodroofe, G. M. (1952): Austral. J. exp. Biol., 30, p. 139.
Green, R. H., Anderson, T. F. and Smadel, J. E. (1942): J. exp. Med., 75, p. 651.
Holmes, F. O. (1948): "Bergey's Manual of Determinative Bacteriology", Sixth Edition. The Williams and Wilkins Co., Baltimore, U.S.A.
Peters, D. and Nasemann, Th. (1952): Naturwiss, 39, p. 306.
Rivers, J. M. and Ward, S. (1937): J. exp. Med., 66, p. 1.
Ruska, H. and Kausche, G. A. (1943): Zbl. Bakt., 150, p. 311.
Sharp, D. G., Taylor, A. R., Hook, A. E. and Beard, J. W. (1946): Proc. Soc. exp. Biol., 61, p. 259.
Smadel, J. E. and Hoagland, C. L. (1942): Bact. Rev., 6, p. 79.

NON-KERATINS OF HUMAN TOENAILS

by A. BOLLIGER AND R. GROSS[1]

(From the Gordon Craig Research Laboratory, Department of Surgery, University of Sydney).

(Accepted for publication 21st November, 1952.)

The organic non-protein substances contained in the aqueous extract of vertebrate keratinous structures and referred to as non-keratins (Bolliger and Gross, 1952) have been identified and determined in a wide variety of keratinous appendages of the skin. It seemed difficult, however, to establish quantitative values for constituents of the non-keratins of human head hair because this keratinous substance is constantly interfered with by grooming. We therefore turned our attention to human toenails because it is obvious that these horny plates so well secured in the nail bed with a comparatively small exposed surface, suffer far less from interference than head hair. Furthermore, it is clear that the secretions of sweat and sebaceous glands would not complicate the results seriously.

MATERIALS AND METHODS.

The toenails of 30 cadavers of persons dying from a wide variety of diseases were examined for a number of non-keratins. Toenail parings of normal living subjects were similarly investigated. The nails were dried by blotting with filter paper and scraped clean with a scalpel. Then after cutting them into small pieces they were extracted with boiling water as described previously. The aqueous extract was examined for total nitrogen, ammonia, urea, uric acid, creatinine, creatine, reducing substances, before and after hydrolysis, pentose and desoxyribose. Due to the small amount of material available in each case, (0·5–2·2 gm.) it was possible only to carry out a few of these determinations on each individual set of toenails. Thus in some instances figures for combined urea and ammonia nitrogen could be obtained only and separate ammonia nitrogen could not be determined. Desoxyribose was determined only in three and preformed creatinine in seven instances.

The analytical methods employed were those described previously (Bolliger and Gross, 1952). In addition to Folin's (1914) "indirect" method for the determination of creatine we used the "direct" diacetyl method (Raaflaub and Abelin, 1950). The figures obtained by the two methods agreed tolerably well. All extracts were also tested with Sakaguchi's qualitative reagent for compounds containing the guanidine group other than creatinine and creatine. In Table 1 uric acid, creatinine and creatine are expressed as such, as customary, and ammonia and urea by their nitrogen content.

RESULTS.

All analytical results from the examination of human toenails are listed in Table 1 with the exception of those from seven patients with marked renal insufficiency or gout which will be discussed elsewhere. The aqueous extracts

[1] Working under a grant from the National Health and Medical Research Council.

of all the nails examined contained considerable amounts of ammonia, urea, uric acid, creatinine, creatine, reducing substances, pentose and desoxyribose. Sakaguchi's reaction was always positive.

TABLE 1.

Diagnosis	Age	Sex	Total N. mg. p.c.	NH_3—N. mg. p.c.	Urea—N. mg. p.c.	Urea + NH_3 – N. mg. p.c.	Uric acid mg. p.c.	Creatinine total mg. p.c.	Creatinine mg. p.c.	Creatine mg. p.c.	Subst. Red. Hydrolysis Before mg.p.c.	Subst. Red. Hydrolysis After mg. p.c.	Pentose mg. p.c.	Desoxyribose mg. p.c.
1. Leukaemia	26	F	742				12				160	195	60	
2. Ca. oesophagus	45	M	410				5				102	148		
3. Ca. breast	52	F				75	6							
4. Cerebral haemorrhage	62	M					7							
5. Peritonitis	58	F	450				6				90		58	
6. Mitral stenosis	48	F	460	14	51	65	7						45	
7. Thrombosis	57	M	460	25	33	58	7							10
8. Acute leukaemia	35	M	464	22	24	46								
9. Acute leukaemia	32	F	448	53	53	106	7							
10. Cerebral tumour	19	F	362	27	38	75								
11. Osteogenic sarcoma	68	F	359	32	29	61	9						15	5
12. Aleukaemic leukaemia	25	M	561								124	174	31	
13. Ruptured gastric ulcer*	55	F	905			112		27						
14. Cerebral tumour	52	M	543	28	31	59								
15. Tuberculosis		M	367	28	16	44			18				53	
16. Cerebral haemorrhage	48	F					9				106		31	
17. Cirrhosis liver	73	F	740					27	18	9				
18. Ca. oesophagus	64	M						37	20	17				
19. Ruptured abdominal aneurism	74	F	360				6	22	11	11	102	134	49	7
20. Ruptured cerebral aneurism	25	F	374			68		30	17	13				
21. Bronchogenic carcinoma	45	M	536			59		30						
22. Cardiac infarct.	63	F	346			65		26	13	13				
23. Cardiac infarct.	67	M	516			44	6	30	15	15				
Normal living. Nail parings.	54	M	376	40	15	55	6						51	
Normal living. Pooled specimen		M	349			38								

* Clubbed nails.

Total nitrogen. This refers to non-protein nitrogen only since no proteins could be demonstrated in the aqueous extract of toenails. The bulk of the total nitrogen values ranges from 350 to 560 mg. p.c. (average 430 mg. p.c.). Exceptions are presented by a patient with clubbed toenails (Case 13, 905 mg. p.c. total nitrogen), one of leukaemia (Case 1, 742 mg. p.c. total nitrogen) and one of cirrhosis of the liver (Case 18, 740 mg. p.c. total nitrogen). With the exception of a patient dying from leukaemia (Case 9, 53 mg. p.c.) *ammonia nitrogen* varied from 14 to 40 mg. p.c. (average 28 mg. p.c.).

Urea nitrogen was present in approximately the same amount as ammonia nitrogen. Excepting Case 9 it varied from 15 to 51 mg. p.c. (average 30 mg. p.c.).

Ammonia and urea nitrogen. With the exception of Cases 9 (leukaemia) and 13 (clubbed toenails) ammonia and urea nitrogen together did not exceed 75 mg. p.c. (average 59 mg. p.c.). This is of interest in those instances where owing to the lack of material separate ammonia determination could not be executed.

Uric acid. Uric acid values as determined by the uricase method varied from 5 and 9 mg. p.c. in extracts of 12 out of 13 specimens. The exception was a case of leukaemia with elevated blood uric acid content. In ten specimens it was 7 mg. p.c. or less.

Creatinine and creatine. Total creatinine varied from 22 to 37 mg. p.c. (average 29 mg. p.c.). Approximately half of this is preformed creatinine and the rest creatine.

Reducing substances. Total reducing substances before and after hydrolysis were determined in a few instances only and varied between 90 and 160 mg. p.c. before hydrolysis. After hydrolysis they were somewhat higher. Pentose was determined on nine specimens and was always present and varied from 15 to 60 mg. p.c. (average 45 mg. p.c.). Desoxyribose was also present but it was determined on three occasions only.

Sakaguchi's test for guanidine compounds other than creatinine and creatine was always positive.

DISCUSSION.

As with other vertebrate keratinous structures studied so far (Bolliger and Gross, 1952) human toenails always yield non-keratin compounds such as ammonia, urea, uric acid, creatinine, creatine, other compounds containing the guanidine group, reducing substances including pentose and desoxyribose. The presence of considerable amounts of urea is characteristic of Mammalia (Bolliger and Gross, 1953). The comparatively low uric acid content of the keratinous appendages is typical of the species *Homo sapiens.*

The customary washing of feet has in all probability little effect on the non-keratin content of the toenails in contrast to the usually more intensive grooming of head hair where readily soluble substances, such as ammonia and urea are practically leached out (Bolliger and Gross, 1952). The large surface presented by head hair in all probability facilitates this leaching process. However, head hair as well as pubic hair washed only scantily or not at all has approximately the same non-keratin content as toenails as will be shown in a subsequent communication.

The bulk of the observations were made on toenails from elderly patients who had died from a wide range of diseases. At this stage, it would be difficult to postulate axiomatically which of the values observed could be considered as within "normal" range. However, if we disregard for the time being toenail extracts with comparatively high urea content from patients with markedly

elevated blood urea levels, it is remarkable how close together lie the analytical values obtained from these diverse cases (Table 1). Furthermore, these findings agree closely with the few observations made on toenail parings from living normals. Tentatively "normal" ranges of figures which apparently apply to adults of all ages would be for total nitrogen 350 to 560 mg. p.c., ammonia 14 to 40 mg. p.c., urea 15 to 50 mg. p.c., uric acid 6 to 9 mg. p.c., total creatinine 22 to 35 mg. p.c., pentose 15 to 50 mg. p.c. A striking exception was found in a set of clubbed toenails which yielded an exceptionally high nitrogen content (905 mg. p.c.). On the other hand, it is realized that the number of observations is far too small to attribute deviations from "normals" to specific diseases.

The amount of total nitrogen present in the aqueous extract of toenails is approximately of the same order as that found in most mammalian furs, birds' feathers, and snake skins, but the gap between determined and undetermined nitrogen is larger in toenail non-keratins than in most other vertebrate non-keratins. Ammonia, urea, uric acid and total creatinine represent less than one-third of the total nitrogen. This probably is due in part to the low uric acid values encountered in toenails. As in other keratinous substances some of the undetermined nitrogen may be accounted for by allantoin, amino acids, purine and guanidine derivatives such as glycocyamine as indicated by a persistently strong Sakaguchi reaction.

SUMMARY.

Total nitrogen, ammonia, urea, uric acid, creatinine, creatine, reducing substances before and after hydrolysis, pentose and desoxyribose were determined on the aqueous extract of human toenails obtained from cadavers of patients dying from a wide variety of diseases and from normal living individuals. In contrast to the groomed head hair, ammonia and urea and other non-keratin constituents of toenails were found to be of approximately the same order as those found in keratinous structures of other mammals. The uric acid values, however, were comparatively low.

Tentative ranges for "normal" values are suggested and a few exceptions presented by clubbed toenails, leukaemia, renal insufficiency, etc., are revealed.

REFERENCES.

Bolliger, A. and Gross, R. (1952): Austral. J. exp. Biol., 30, p. 181.
Bolliger, A. and Gross, R. (1953): In process of publication.
Raaflaub, J. and Abelin, I. (1950): Biochem. Z., 321, p. 158.

THE METABOLISM OF THE SEMINAL VESICLE OF THE GUINEA-PIG

I. RESPIRATION

by G. F. HUMPHREY AND MARJORY ROBERTSON[1]

(From the Department of Biochemistry, University of Sydney).

(Accepted for publication 29th October, 1952.)

Many studies have been made of the metabolic activities of mammalian spermatozoa both for the intrinsic interest of such studies and also for the practical value of the results obtained (Mann, 1949). It is often important to obtain an assessment of the viability and metabolic potential of spermatozoa, and biochemical methods devoted to these ends examine both respiration and glycolysis. Epididymal or ejaculated spermatozoa can be used, and in the latter instance the cells are usually separated from the seminal plasma and washed two or three times before use. *In vivo,* however, the sperm functions in close co-operation with the constituents of the seminal plasma. This is so because ejaculated semen is a closely integrated system of cells and fluid, the former depending on the latter for their well-being and the latter representing the product of another well integrated system, i.e. the accessory reproductive tissues. These produce their characteristic secretions which are added to the sperm at the appropriate moment, providing nutrients, co-factors, enzymes, etc., which all play a part in the process of reproduction.

The concentrations of the constituents which make up the seminal plasma are under the control of the nutritional and endocrine states of the organism. These controlling factors exert their influence upon the activities of the accessory tissue and hence determine the composition of the seminal plasma. Very little is known about the metabolism of accessory male reproductive tissue, especially the seminal vesicle, an organ which is capable of secreting high concentrations of unusual constituents. The present investigation is concerned with the general respiratory activity of guinea-pig seminal vesicle; this preliminary background is needed to evaluate some of the unusual metabolic features of this tissue.

MATERIALS AND METHODS.

Seminal vesicles were taken from freshly-killed animals; the glands were freed from other tissue and the gel content expelled with forceps. The gland was then slit longitudinally

[1] Working under a full-time grant from the National Health and Medical Research Council. This investigation was also supported by apparatus grants from the Cancer Research Committee, University of Sydney.

and cut into pieces about 1 cm. long. These "slices" were then washed by immersion for a few minutes in ice-cold Ringer solution (Ca-free, prepared according to Mann, 1945).

Cytochrome *c* was prepared according to Keilin and Hartree (1937) and standardised spectrophotometrically by the method of Umbreit *et al.* (1945). Commercial pyruvic acid was distilled *in vacuo*, diluted tenfold and stored at —15° C. Other compounds used were commercial specimens, chemically pure.

Homogenates were prepared by grinding the tissue in a stainless steel micro Waring blendor or between glass surfaces (Humphrey, 1946). Both types of instruments were fitted with cooling jackets containing alcohol at —10° C. and at the end of the grinding period the temperature of the tissue preparation was between 2 and 5° C.

KCl homogenates were made with ice-cold 0·9 p.c. KCl containing enough KOH to keep the pH approximately neutral. For two pairs of seminal vesicles a total of 30 ml. of KCl and 2 drops of 20 p.c. KOH was found to be satisfactory. Either the whole homogenate was used or a washed preparation made by centrifuging at 0° C. for 15 minutes at 2,000 × *g.*, discarding the opalescent supernatant (containing some mitochondria) and washing the residue twice by centrifuging for 7 minutes after stirring with about 5 volumes of 0·9 p.c. KCl; the residue was then suspended in Ringer —0·01 M phosphate.

Tissue fractions were prepared at 0° C. from homogenates in 30 p.c. sucrose (Hogeboom *et al.*, 1948). The sucrose homogenate (H) was centrifuged for 3 minutes at 1,500 × *g.* and the residue washed twice with 3 volumes of sucrose. The residue was suspended in Ringer solution and represents the nuclear fraction (N). The combined supernatants were centrifuged for 20 minutes at 18,000 × *g.* and the supernatant preserved as the "soluble" fraction (S). The residue was resuspended in 10 volumes of sucrose and centrifuged as before; the residue is the mitochondrial fraction (M) and was suspended in Ringer solution.

Incubation was carried out aerobically at 37° C. in Warburg flasks. At the end of the incubation period (1 or 2 hours) an equal volume of 16 p.c. trichloracetic acid was added. The dry weight of tissue preparations was determined by heating to constant weight at 80° C., and total nitrogen by incineration with concentrated H_2SO_4 and H_2O_2, followed by direct Nesslerization. Pentose and desoxypentose nucleic acids were extracted from tissues and experimental mixtures according to both the long and short procedures of Schneider (1945) and the sugars estimated according to Morse and Carter (1949).

EXPERIMENTAL.

Composition of homogenates and tissue fractions.

The tissue preparations were examined microscopically by staining and also by the phase contrast technique. For staining, material was mixed with an equal volume of 0·02 p.c. Janus green B, 0·1 p.c. fast green F.C.F. or 0·1 p.c. toluidine blue. As a routine, high-power phase contrast examination of unstained preparations was found most satisfactory.

When using the Waring blendor, the activity of the various suspensions was influenced both by the speed and duration of blending. Preliminary experiments showed that good activity was given after 30 to 60 seconds at both 12,000 and 15,000 r.p.m. However, the higher speed often gave preparations from which intact nuclei and mitochondria could not be recovered in good yield and possessing good enzymic activity. Therefore, 12,000 r.p.m. was chosen as the speed of blending; 60 seconds were needed to give satisfactory preparations of cell constituents. With the ground glass homogenizer, a constant speed of 450 r.p.m. was used for 2 minutes.

The KCl homogenates contained tissue debris, nuclei and some of the mitochondria. In the tissue fractions, N contained tissue debris, nuclei and a few mitochondria, M only mitochondria and S the "soluble" constituents and occasionally a few mitochondria. On the average, 60 p.c. of the total nitrogen of the sucrose homogenate could be recovered in the N, M and S fractions, i.e. about 40 p.c. was lost in the washing procedure.

TABLE 1.

Composition of fractions.

	H	N	M	S
		Waring Blendor		
mg. desoxypentose	6·0	1·5	1·0	4·0
mg. pentose	1·2	0·4	0·4	0·3
		Glass Homogenizer		
mg. desoxypentose	5·5	4·4	0·1	0·1
mg. pentose	1·5	0·6	0·2	0·2

TABLE 2.

Composition of KCl Homogenates.

	Supernatant	First washing	Second washing	Final suspension
		Waring Blendor		
mg. desoxypentose	1·4	0·9	0·5	2·3
mg. pentose	0·10	0·02	0	0·70
		Glass Homogenizer		
mg. desoxypentose	0	0	0	4·4
mg. pentose	0·2	0	0	1·0

As an additional check on the cytological purity of the preparations, chemical analyses for pentose and desoxypentose compounds were carried out. These showed that when the Waring blendor was used, significant amounts of desoxypentose compounds occurred in fractions other than the nuclear one. In the estimation, the short extraction method gave higher results than the long extraction method indicating that compounds simulating desoxypentose were removed by the more laborious and numerous treatments in the latter. Since it was apparent that treatment with the blendor ruptured some of the nuclei thus liberating desoxypentose compounds which appeared in other fractions or wash fluids, the use of the blendor was abandoned in favour of the glass homogenizer. In this the tissue is squashed, not minced. With this milder method, good separation of the tissue components was obtained and the desoxypentose compounds of the homogenate were recovered in the nuclear fraction and not spread through the fractions (mainly the soluble one) as with the blendor. Table 1 shows the

type of results obtained using the long extraction method for pentose and desoxypentose; complete recovery of the various constituents originally present in the homogenate cannot be obtained owing to the washing procedures used.

With regard to the KCl homogenates, Table 2 shows that the blendor has a similar action here and that desoxypentose is lost from the nuclei and appears in the washings. The use of the glass homogenizer does not rupture the nuclei.

Respiration.

Slices. A few experiments were carried out with slices of seminal vesicle and the results are shown as p.c. of the endogenous O_2 uptake in Table 3. Here the slices were blotted dry and quickly weighed on a torsion balance; each slice weighed between 150 and 250 mg. and was suspended in 1 ml. Ringer solution plus 0·1 ml. 0·2 M phosphate, pH 7·2 and 0·1 ml. 0·1 M neutralized substrate.

TABLE 3.

Respiration of slices.

	Values are p.c. of endogenous O_2 uptake	
Substrate	Range	Mean
Citric acid	100–150	120
Succinic acid	100–200	130
Fumaric acid	100–110	103
Malic acid	110–150	120
Acetic acid	80–110	100
Propionic acid	100–150	120
Butyric acid	100–160	125

Homogenates. The results for washed and whole KCl homogenates are given in Table 4. In all cases 0·01 M phosphate, pH 7·2 was present.

TABLE 4.

Respiration of Homogenates.

	Whole homogenate		Washed homogenate	
Substrate	Range	Mean	Range	Mean
Cytochrome	100–140	130	100–130	125
Pyruvic acid	100–200	140	130–200	160
Citric acid	100–200	155	120–180	150
α-ketoglutaric acid	200–250	210	200–400	320
Succinic acid	200–600	420	380–3,000	760
Fumaric acid	100–170	140	150–200	165
Malic acid	100–180	160	200–300	220
Oxaloacetic acid	110–150	135	130–200	145
Acetic acid	120–200	140	60–140	105
Propionic acid	90–150	105	50–140	90
Butyric acid	120–200	140	100–140	125

Tissue fractions. Table 5 contains the results obtained with the tissue fractions. Each experiment was carried out in the presence of 0·01 M phosphate.

TABLE 5.

Respiration of tissue fractions.

	H		N		M		S	
Substrate	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Cytochrome	100–150	120	100–200	160	120–200	170	120–180	165
Pyruvic acid	100–230	180	110–180	135	110–200	175	110–210	180
Citric acid	250–350	270	200–300	220	60–80	75	90–120	105
α-ketoglutaric acid	110–240	190	140–200	165	120–200	165	130–220	170
Succinic acid	210–1,000	560	350–450	375	300–900	720	130–200	150
Fumaric acid	200–310	230	160–220	180	60–80	75	80–100	95
Malic acid	250–350	275	200–300	255	30–60	50	110–140	120
Oxaloacetic acid	100–120	115	100–210	145	100–130	110	120–150	135
Acetic acid	100–180	135	120–150	130	100–120	105	140–180	155
Propionic acid	180–250	200	110–120	115	110–150	120	120–160	130
Butyric acid	150–250	170	100–120	105	100–120	105	100–130	110

TABLE 6.

Keto-acid production by slices and KCl homogenates.

	Slices		Whole homogenate		Washed homogenate	
Substrate	Range	Mean	Range	Mean	Range	Mean
Cytochrome	—	—	150–300	220	120–170	155
Citric acid	80–100	85	130–180	175	120–200	180
Succinic acid	70–100	80	160–200	170	120–210	145
Fumaric acid	100–150	120	270–400	330	140–300	210
Malic acid	100–120	115	350–480	425	120–480	315
Acetic acid	70–100	80	120–170	135	100–150	110
Propionic acid	60–100	70	120–150	130	100–130	105
Butyric acid	50–100	65	100–160	120	100–140	110

TABLE 7.

Keto-acid production by tissue fractions.

	H		N		M		S	
Substrate	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Cytochrome	150–470	300	130–230	160	210–400	290	200–320	280
Citric acid	300–450	350	150–250	185	50–100	80	80–130	105
Succinic acid	150–1,000	475	390–600	480	130–200	160	120–170	155
Fumaric acid	180–360	240	200–260	220	130–200	150	160–230	190
Malic acid	180–320	230	220–300	270	140–220	175	150–220	175
Acetic acid	70–150	120	40–120	80	100–120	105	100–160	140
Propionic acid	50–100	85	20–120	75	80–120	110	20–80	50
Butyric acid	60–130	110	20–130	70	70–120	80	50–100	60

Production of keto-acids.

During incubation, the various tissue preparations form keto-acids and these accumulate in the medium. Tables 6 and 7 show the extent of this accumulation by the different tissue suspensions; the experiments were carried out under the same conditions as before and the values are given as p.c. of the endogenous production. The amount of keto-acids is calculated in terms of pyruvic acid.

Early experiments with tissue fractions using the method of Friedemann and Haugen (1943), indicated that α-ketoglutaric acid was accumulating. This conclusion was based upon the ratios of the optical densities (D) of the 2:4 dinitrophenylhydrazones at 400 and 420 mμ, compared to the value at 540 mμ.

Further experiments with KCl homogenates and tissue prepared by other methods showed that the ratios obtained were often much lower, approaching those indicating pyruvic acid. In view of the fact that Friedemann and Haugen used a filter photometer with a wide bandwidth to determine the absorption data and the present study was made with a Beckman model DU spectrophotometer with a narrow bandwidth of only a few mμ., it was thought best to re-examine the absorption characteristics of 2:4 dinitrophenylhydrazones of keto-acids of biological importance.

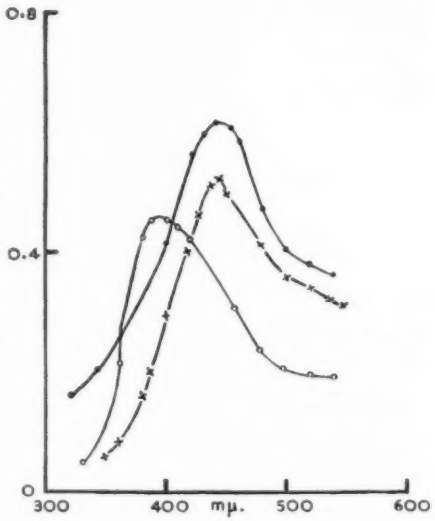


Fig. 1. Optical density of 2:4 dinitrophenylhydrazones at different wave-lengths.
● oxaloacetic acid
○ α-ketoglutaric acid
× pyruvic acid

Absorption curves of 2:4 dinitrophenylhydrazones.

Fig. 1 shows the curves obtained in the present investigation. The hydrazones were formed under the conditions of estimation, i.e. reaction of a small quantity (50 μg.) of keto-acid with excess reagent, extraction with ethylacetate and then colour development in 0·75 M NaOH in 0·5 M Na_2CO_3. Table 8 gives a comparison of the values obtained with those of Friedemann and Haugen (1943), Cavallini (1950) and LePage (1950). Cavallini and LePage used M NaOH for colour development and the values tabulated are calculated from the absorption curves which they obtained.

TABLE 8.

Absorption values of 2:4 dinitrophenylhydrazones.

		Friedemann and Haugen	Cavallini	LePage
Pyruvic acid.				
Maximum, mμ.	445	425	443	445
D400/D540	0·90–1·05	1·20	0·95	—
D420/D540	1·30–1·41	1·46	1·33	1·40
Oxaloacetic acid.				
Maximum, mμ.	440–442	425	455	450
D400/D540	1·10–1·25	1·20	0·92	—
D420/D540	1·36–1·60	1·46	1·17	1·20
α-ketoglutaric acid.				
Maximum, mμ.	390–400	below 400	422	432
D400/D540	2·09–2·51	2·25	1·85	—
D420/D540	1·95–2·36	2·07	2·00	1·60

Although Cavallini and LePage used the same solvent for colour development and the same type of spectrophotometer (Beckman) their results differ. As shown above, the results obtained in the present investigation were subject to slight variation. The results cover experiments in which keto-acids were dissolved in trichloracetic acid and those in which keto-acids were added to preparations of seminal vesicle which had been treated with trichloracetic acid immediately after preparation. Both these types of experimentation were used to see if any modification of the absorption pattern was caused by substances extracted from the tissue; no such interaction was observed.

The hydrazones are not completely stable in alkaline solution and on standing there is a progressive loss in optical density, but no change in the position of maxima. Following the recommendation of Friedemann and Haugen (1943) the readings were commenced 5 to 10 minutes after formation.

In summary then, it can be stated that initially it was thought (following Friedemann and Haugen) that the keto-acids could be characterized by the values of the ratios of their densities, but since these values are somewhat variable the position of the absorption maximum must also be noted. The acids accumulating have provisionally been identified as one of the three whose absorption curves have been studied; it is possible that the data would fit other compounds. With these assumptions, it seems that the following conclusions can be drawn—slices of tissue accumulate a mixture of oxaloacetic and pyruvic acids, KCl homogenates oxaloacetic acid only and tissue fractions α-ketoglutaric acid with sometimes a trace of pyruvic or oxaloacetic acid. Specimen results are set out in Table 9.

TABLE 9.

Identification of keto-acids produced by tissue preparations.

Preparation	Absorption maximum	D400/D540	D420/D540	Nature of acids
slices	440 = 443 = 445 mμ	1·02	1·38	pyruvic + oxaloacetic
KCl homogenate	440	1·10	1·52	oxaloacetic
Nuclei	400	2·10	1·95	ketoglutaric
Nuclei	400	1·88	1·73	ketoglutaric + others
Nuclei	430	1·50	1·88	ketoglutaric + others

DISCUSSION.

Composition of preparations.

The results show quite clearly that the Waring blendor ruptures some of the cell constituents and that the gentler method of grinding between glass surfaces preserves the components and does not release desoxypentose compounds from the nuclear fraction. The ratio of the amount of desoxypentose to pentose in the seminal vesicle is about 4 to 1. This ratio is similar to that found in thymus, mouse lung and sheep spleen, but different from that in most animal tissues (Davidson, 1947). However, as Logan *et al.* (1952) have shown, the amount of and ratio between these two compounds depends on the method of estimation and caution should be exercised in interpreting any results. The KCl preparations are essentially a mixture of all the nuclei and some of the mitochondria. The presence of mitochondria in such preparations is a result of their agglutination by the electrolyte (Hogeboom and Schneider, 1950), and this allows sedimentation at relatively low speeds.

Respiration.

The respiration of slices of tissue was found not to be very sensitive to added substrates and this type of preparation was soon discarded; permeability factors are probably the cause of the lack of effect because when the tissue is broken up the respiration is increased by many added compounds.

Table 10 shows the relationship between the endogenous respiration of the various preparations. The sucrose fractions varied greatly in activity from one experiment to another, but always the nuclear fraction was the least active and the mitochondria and soluble constituents the most active.

The KCl homogenates were more constant in their ability to take up oxygen and the activity fell off somewhat on washing, due probably to the removal of mitochondrial and soluble constituents. Slices exhibited a very uniform, active respiration. Homogenates prepared in Ringer's solution did not respire very much.

TABLE 10.

Effect of method of preparation on respiration and keto-acid production.

	Q_{O_2}/mg.N		$Q_{\text{keto-acids}}$ /mg.N	
	Range	Mean	Range	Mean
Slices	4·0–5·0	4·6	0·5–3·0	1·2
Whole homogenate	4·0–7·0	5·2	3·0–5·0	4·2
Washed homogenate	2·0–6·0	3·8	2·0–6·0	4·1
H	0·7–7·0	3·5	1·4–7·0	2·8
N	0·5–2·5	1·2	0·3–1·3	0·7
M	3·0–26·0	10·8	0·2–3·5	1·4
S	2·0–16·8	7·0	0·7–4·8	3·0

Cytochrome *c* stimulated all preparations but not very markedly. Keto-acids could double the respiration of all preparations and fatty acids had little or a moderate effect, their action being most marked on the sucrose homogenate. Succinic acid powerfully stimulated respiration in all cases; among the tissue constituents the mitochondria showed the greatest relative effect. Fumaric and malic acids produced a fall in respiration of the mitochondria but an increase with the nuclei.

Previous study of the respiration of seminal vesicle is confined to the observations of Mann and Lutwak-Mann (1948). These workers used slices of rat seminal vesicle and reported a $Q_{O_2} = 2$ per mg. dry weight; this would correspond to about 8 per mg. nitrogen. With regard to other accessory tissue, prostate has been studied by Barron and Huggins (1944 and 1946) and using slices of dog, rabbit and human prostate these workers found values of the Q_{O_2} ranging from 2–6 per mg. dry weight, corresponding to about 8–24 per mg. nitrogen. Only succinic acid had any significant effect in raising the level of respiration (an increase of 70 p.c.). These values for respiratory intensity are similar to those obtained in the present investigation.

As a conclusion it may be said that the endogenous respiration of the tissue is brought about by the mitochondrial and soluble constituents. As judged by the oxygen consumption, the activity towards succinic acid is in the mitochondria and that towards fumaric and malic acids is in the nuclear fraction. The KCl homogenates show activity to all these compounds because they combine the nuclear and mitochondrial portions.

Keto-acid production.

Fumaric and malic acids were the most active compounds in stimulating keto-acid production. Succinic acid was very active except where it produced the greatest increases in respiration. In other words there was least accumulation of by-products where the increase in respiration was largest. With the mitochondria and soluble constituents, which both showed decreased respiration in the presence of fumaric and malic acids, there was nevertheless an increase in keto-acid production.

SUMMARY.

An examination was made of the preparation of seminal vesicle for metabolic studies. The use of the Waring blendor was discontinued as it destroys some of the nuclei and permits the escape of desoxypentose compounds. Glass homogenizers preserve the structure of the nuclei and were therefore used.

The ratio of desoxypentose to pentose in seminal vesicle preparations is about 4 to 1.

The mitochondria and "soluble" constituents of the seminal vesicle are responsible for the greater part of the endogenous respiration. Several compounds stimulate respiration: fumaric and malic acids exert their effect mainly on the nuclear fraction and succinic acid mainly on the mitochondria.

Keto-acids accumulate during respiration, the soluble constituents being most active in this respect.

REFERENCES.

Barron, E. S. G. and Huggins, C. (1944): J. Urol., 51, p. 630.
Barron, E. S. G. and Huggins, C. (1946): *Ibid.*, 55, p. 385.
Cavallini, D. (1950): Ric. Sci., 20, p. 803.
Davidson, J. N. (1947): Symposium Soc. exp. Biol., 1, p. 77.
Friedemann, T. E. and Haugen, G. E. (1943): J. biol. Chem., 147, p. 415.
Hogeboom, G. H., *et al.* (1948): *Ibid.*, 172, p. 619.
Hogeboom, G. H. and Schneider, W. C. (1950): *Ibid.*, 186, p. 417.
Humphrey, G. F. (1946): Austral. J. exp. Biol., 24, p. 261.
Keilin, D. E. and Hartree, E. F. (1937): Proc. roy. Soc. Lond. Ser. B, 122, p. 298.
LePage, G. A. (1950): Cancer Res., 10, p. 393.
Logan, J. E., *et al.* (1952): Biochem. J., 51, p. 470.
Mann, T. (1945): *Ibid.*, 39, p. 451.
Mann, T. (1949): Adv. Enzymol., 9, p. 329.
Mann, T. and Lutwak-Mann, C. (1948): Biochem. J., 43, p. 266.
Morse, M. L. and Carter, C. E. (1949): J. Bact., 58, p. 317.
Schneider, W. C. (1945): J. biol. Chem., 161, p. 293.
Umbreit, W. W., *et al.* (1945): "Manometric Techniques and related Methods". Burgess Publishing Co., Minneapolis.

THE METABOLISM OF THE SEMINAL VESICLE OF THE GUINEA-PIG

II. CITRIC ACID BREAKDOWN

by G. F. HUMPHREY AND MARJORY ROBERTSON[1]

(From the Department of Biochemistry, University of Sydney).

(Accepted for publication 29th October, 1952.)

Citric acid occurs in mammalian seminal plasma in concentrations as high as 1 p.c. Depending on the particular organism, it is produced by the seminal vesicle, the prostate or both of these tissues. Its production is consequent upon normal functioning of those systems concerned with testosterone, gonadotrophins and vitamin B. Although no satisfactory rôle for citric acid in the process of reproduction has yet been advanced, possibilities are in relation to the coagulation-liquefaction phenomenon of semen, the respiratory pathways and the control of testosterone levels by systems which need citric acid as a co-factor (Sweat and Samuels, 1948).

Exploratory experiments confirmed that the seminal vesicle of the guinea-pig produces a gel-like secretion containing a high concentration of citric acid, and the problem under investigation is how an important metabolite such as citric acid is able to accumulate in accessory reproductive tissue when most other tissues convert this compound to other products. The ability of seminal vesicle tissue to decompose citric acid was therefore first examined.

MATERIALS AND METHODS.

The materials and methods used were those of the previous investigation (Humphrey and Robertson, 1953) with the following additions.

Citric acid was estimated by the method of Weil-Malherbe and Bone (1949), and fructose by the method of Roe (1934).

$Q_{citrate}$ is calculated per mg. N.

Anaerobic incubation was carried out in Thunberg tubes filled with N_2 freed from O_2 by passage over Cu turnings at 350° C.

EXPERIMENTAL.

Distribution of citric acid.

Previous studies (Humphrey and Mann, 1948) have shown that in the rat citric acid is produced by accessory male reproductive tissue different from that

[1] Working under a full-time grant from the National Health and Medical Research Council. This investigation was also supported by apparatus grants from the Cancer Research Committee, University of Sydney.

which produces fructose. Citric acid was found in the seminal vesicle and ventral prostate whereas fructose occurred in the coagulating gland and median and dorsal prostates. This is not the case in the guinea-pig; there the seminal vesicle is responsible for the production of most of the citric acid as well as of the fructose (Table 1). In these experiments the organs were dissected out, quickly weighed and ground with a small quantity of sand. The mixture was then extracted twice with 8 p.c. trichloracetic acid.

TABLE 1.

Citric acid and fructose in accessory reproductive organs.

Tissue	Weight (gm.)	Citric acid (mg. p.c.)	Fructose (mg. p.c.)
Testis	3·25	19·7	1·5
Vas deferens	0·44	14·5	7·2
Seminal vesicle			
secretion	2·77	210·0	63·0
gland	1·68	30·0	32·8
Prostate, dorsal	0·39	83·0	18·1
Prostate, ventral	0·57	106·0	15·8

There was no significant difference between the dorsal and ventral lobes of the prostate with regard to their content of either citric acid or fructose.

Breakdown of citric acid.

Various methods of tissue preparations were used (Humphrey and Robertson, 1953) to discover how much the extent of citricolysis varied with the state of the tissue. Experimental conditions were also varied to see what was their influence.

Effect of tissue concentration. These experiments (Table 2) were carried out in the presence of 0·002 M citrate in 0·01 M phosphate, pH 7·2. The tissue was prepared by blending in Ringer-phosphate for 30 seconds at 12,000 r.p.m. It can be seen that as the amount of tissue was decreased, the extent of citricolysis increased.

TABLE 2.

Tissue concentration and citricolysis.

	$Q_{citrate}$	
mg. dry tissue/ml.	Aerobic	Anaerobic
2·8	11·2	8·8
5·6	4·4	4·4
11·2	2·3	1·4
22·4	1·5	1·3

Effect of citrate concentration. A blended preparation in Ringer phosphate was used in this series and the final reaction mixture contained 3 mg. dry tissue per ml. Increasing the citrate concentration brought about an increase in the citricolysis (Table 3).

TABLE 3.

Citrate concentration and citricolysis.

Citrate concentration	$Q_{citrate}$ Aerobic	Anaerobic
0·001M	0·4	0·4
0·002	4·8	4·8
0·005	6·7	6·4
0·010	14·0	13·4

Effect of pH. The action of pH was studied on slices (Table 4), KCl homogenates (Table 5), and tissue fractions (Table 6). Only a few experiments were carried out with slices (suspended in Ringer —0·01 M phosphate —0·01 M citrate), because it was shown (Humphrey and Robertson, 1953) that this type of preparation did not respond to added substrates so well as homogenates and subcellular fractions.

With homogenates, the amount of citricolysis was greater than with slices, washed homogenates being about twice as powerful as whole homogenates. The effect of pH was not very marked nor was there usually a great difference between anaerobic and aerobic breakdown. The experiments were carried out in the presence of 0·01 M phosphate and 0·01 M citrate.

TABLE 4.

Citricolysis by slices.

pH	$Q_{citrate}$ Aerobic	Anaerobic
6·4	3·88	2·72
7·2	3·96	1·76
7·8	3·80	1·64
8·6	4·02	1·88

TABLE 5.

Citricolysis by washed homogenates.

pH	Aerobic	Anaerobic
6·4	22·0	13·2
7·2	22·0	13·8
7·8	21·1	10·8
8·6	19·6	7·4

Using tissue fractions prepared from sucrose homogenates, the whole homogenate (H) was compared with the nuclear (N), mitochondrial (M) and "soluble" fractions (S). Again, 0·01 M phosphate and citrate were used, and under anaerobic conditions, the optimum values were found to be at a more alkaline level than those obtained aerobically.

TABLE 6.

Citricolysis by tissue fractions.

	H		N		M		S	
pH	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
6·4	7·6	3·8	9·4	12·7	12·5	5·6	7·4	1·1
7·2	12·0	7·5	6·4	12·7	39·0	9·4	27·3	14·4
7·7	9·1	12·9	2·3	22·8	20·3	10·0	23·6	21·5
8·6	8·2	1·7	3·5	15·9	22·0	21·9	12·4	30·3

DISCUSSION.

Citricolysis.

Five types of tissue preparations were used in these experiments and these types may be divided into two classes:

1. Little preliminary treatment: slices, whole homogenates in Ringer's solution and whole homogenates in KCl.
2. Extensive preparation: washed KCl homogenates and sucrose fractions.

The extent of citricolysis shown by preparations of the first class is small ($Q<15$) and it could be concluded that the seminal vesicle can bring about only a small amount of citrate breakdown. However, with the methods of class 2 the values of the $Q_{citrate}$ can exceed 30 when the active cell constituents (those in fractions M and S) are separated and used alone. These increases in activity may be due simply to a removal of inert material or to a change in the pattern of metabolism brought about by a reduction in the interaction of several competing functional units. These more active units are also those in which the aerobic breakdown is, on the whole, higher than that under anaerobic conditions.

With all types of tissue preparation, the effect of pH varied from one experiment to another; there was no clearly marked pH optimum. This variation probably results from the composite nature of the process being studied.

There are no values in the literature concerning the breakdown of citric acid by seminal vesicle. With ram sperm, Humphrey and Mann (1949) found that the $Q_{citrate}$ was about 0·15 per mg. dry weight and also that bull seminal plasma contained a heat-labile factor which inhibited the breakdown of citric acid by otherwise active tissues, e.g. liver. Barron and Huggins (1946) state that prostatic tissue is very deficient in ability to decompose citric acid, the Q

value being about 1·4 and conclude therefore that the accumulation of citric acid by this tissue is aided by the lack of breakdown once the compound is formed.

It can be concluded that the mitochondrial and soluble constituents are the units in the cells of the guinea-pig seminal vesicle which are responsible for the breakdown of citric acid and that the activity of other preparations is largely if not entirely due to these. The degree of citric acid synthesis by this tissue must next be examined before the accumulation of citric acid in the seminal vesicle gel can be explained as due to deficient breakdown.

SUMMARY.

Citric acid can be broken down by guinea-pig seminal vesicle. The $Q_{citrate}$ / mg. N of slices is about 4 aerobically and 2 anaerobically; pH has little effect. Using tissue blended in Ringer solution the Q can reach 14 aerobically and anaerobically; with tissue blended in KCl solution the values are of the same order but the aerobic breakdown is slightly more than the anaerobic. Using nuclear, mitochondrial and soluble fractions of the tissue it is found that the nuclear fraction exhibits a rate of breakdown similar to that of the slices (except that anaerobic is greater than aerobic decomposition), but that the other fractions are much more active giving $Q_{citrate}$ values of up to 39, aerobic being greater than anaerobic breakdown.

REFERENCES.

Barron, E. S. G. and Huggins, C. (1946): J. Urol., 55, p. 385.
Humphrey, G. F. and Mann, T. (1948): Nature, 161, p. 352.
Humphrey, G. F. and Mann, T. (1949): Biochem. J., 44, p. 97.
Humphrey, G. F. and Robertson, M. (1953): Austral. J. exp. Biol., 31, p. 131.
Roe, J. H. (1934): J. biol. Chem., 107, p. 15.
Sweat, M. L. and Samuels, L. T. (1948): *Ibid.*, 175, p. 1.
Weil-Malherbe, H. and Bone, A. D. (1949): Biochem. J., 45, p. 377.

STUDIES ON THE PATHOGENESIS OF A HITHERTO UNDESCRIBED VIRUS (*HEPATO-ENCEPHALOMYELITIS*) PRODUCING UNUSUAL SYMPTOMS IN SUCKLING MICE

by N. F. STANLEY, D. C. DORMAN AND JOAN PONSFORD

(From the Institute of Epidemiology and Preventive Medicine, Prince Henry Hospital, Sydney).

(Accepted for publication 1st December, 1952.)

In the course of studying viruses capable of infecting suckling mice (Stanley *et al.*, 1951) numerous samples of faeces were tested for the presence of Coxsackie and poliomyelitis viruses. During this investigation a control series was selected from the surgical wards of the hospital and ultracentrifuge deposits of faecal suspensions, sterilized with a penicillin-streptomycin mixture, were inoculated into litters of suckling mice less than 48 hours old. The inoculation with the faeces of one patient resulted in the isolation of an infective agent which caused an unusual and striking condition in suckling mice that is believed not to have been described elsewhere. It is the purpose of this communication to describe the isolation and some of the properties of this agent.

EXPERIMENTAL.

Case history.

The patient was an aboriginal male child aged 2½ years and resident at Yarra Bay Aboriginal Settlement, Sydney. When three months old the infant was admitted to hospital with whooping cough. At the age of 15 months he was admitted to this hospital on 20/2/51 with a cough, a fever of three days' duration, vomiting and with enlarged and inflamed tonsils. The final diagnosis was "bilateral bronchopneumonia". He was discharged on 1/3/51. He was readmitted on 3/4/51 with bronchopneumonia and bronchiectasis, which was relieved by treatment. The child was recently readmitted to this hospital (March, 1952) for investigation because of the nature of the laboratory findings described in this communication. The child was undernourished and had diffuse bronchitic signs in both lungs. There were three small areas of alopecia on top and one on the occipital region of the head. They were irregular in shape, but approximately one inch in diameter. There was a mild bilateral conjunctivitis. Further specimens of faeces and blood were collected for virus studies. The leucocyte count was normal. Fungi could not be detected in the scrapings from areas of alopecia. Wassermann reaction was negative. X-ray showed some emphysema and bronchitic changes.

Treatment consisted of a generous diet with vitamins. On discharge after 14 days the hair was beginning to grow. A nurse from the aboriginal settlement where the patient resided stated that for some considerable time the hair used to regrow but was followed by new areas of alopecia.

Primary isolation of the agent.

The ultracentrifuge deposit from faeces collected on 22/2/51 was inoculated by the intraperitoneal route into two litters of suckling mice, one on 15/11/51 and one on 7/5/52, with similar results. The patient's faeces had thus been kept at —23° C. for fifteen months without inactivation of the agent. The incubation period was sixteen days, at which time the mice were emaciated, unco-ordinated and showed a peculiar hair effect, associated with an oily secretion which covered the entire body. A detailed description of this effect is given in the following section of this paper.

Infection in Suckling Mice.

Responses to infection:

Oily hair effect (*OHE*). The oily substance covered the entire body so that the hair became matted together in strands revealing the skin. This was very marked on the back of the head, over the sacrum and on the belly (see Figs. 1 and 2).

It was found that when one 12-day old suckling mouse showing OHE was placed for one hour with a normal litter of the same age the oily exudate was rubbed off the hair of the infected mouse by the non-infected ones, including the mother. Thus a mouse showing OHE need not necessarily be infected. As it is believed that there is a fairly high mortality rate among suckling mice infected with the virus it is likely that mice showing OHE only slightly and with no other symptoms are not infected or have a subclinical infection. Following intraperitoneal inoculation, the time of onset of OHE is usually ten to twelve days. A similar incubation period was noted when infant mice were dipped in a suspension of infected tissue. If only a small proportion of the litter was inoculated, the uninoculated litter mates showed OHE and encephalitis and the virus could be recovered from their tissues. Although approximately 30,000 suckling mice have been inoculated in this laboratory within the last two years, the OHE has only been associated with the inoculation of the treated faecal suspension of this one patient.

Encephalitis. The symptoms associated with infection of the central nervous system were observed in a large number of mice. The mice showed emaciation, unco-ordination, tremors and paralysis prior to death. In the early passages ataxia, paralysis and death usually followed the appearance of the OHE, but, with continued passage, the OHE appeared less frequently and the mice developed a fatal encephalitis five to seven days after inoculation.

Jaundice. Yellow ears were the first sign of jaundice in mice, and this colour might extend to the feet, nose or tail. Post-mortem examination revealed even distribution of the pigment over the skin, and it was clearly seen

Fig. 1. Mother and two suckling mice (14 days old) showing OHE. Hair of mother is oily due to contact with young.

Fig. 2. 14-day old suckling mice. The mouse on the right shows OHE following inoculation by the intraperitoneal route 13 days previously with the hepato-encephalomyelitis virus.

Fig. 3. Dorsal view of two litter mates aged 9 days. The mouse on the right shows distended belly due to blood-stained peritoneal exudate.

in the urine. The following figures for total bilirubin were obtained from jaundiced mice:

2·1 mg./100 ml. blood; 0·85 mg./100 ml. peritoneal exudate.

The figure for blood from normal mice of the same age was 0·2 mg. bilirubin/100 ml. Bile pigments were also present in the urine. At no time could carotenoid pigment be detected.

Peritoneal exudate. This usually occurred before the onset of other symptoms and caused great distension of the peritoneal cavity (see Fig. 3). The volume might be as great as 1 ml. in a mouse 7 days old. In appearance it was usually bloodstained or bright yellow. The peritoneal exudate was frequently observed following inoculations by the intraperitoneal or intrahepatic routes, but less frequently when the intracerebral route was used.

Alopecia. This effect was marked but occurred only in those mice recovering from a severe infection whose symptoms included the OHE and encephalitis. Loss of hair was marked on the dorsal area and bald patches were observed on the head, at the back of the neck and near the hind legs. After a period, ranging from ten to fifteen days, the hair commenced to regrow until no sign of alopecia remained, but the animals were greatly retarded in growth. At the time of regrowth of hair some animals developed marked jaundice again and died.

Condition resembling conjunctivitis. This was seen in mice at all stages of infection but was not regular in its appearance.

Course of infection.

Following intraperitoneal inoculation: Virus was demonstrated in the skeletal muscle, the liver, the brain, the peritoneal exudate and the lung. To study the distribution of the virus in the brain and liver during the course of infection mice were selected at 48-hour intervals from four inoculated litters and their brains and livers titrated. At the same time material was kept for histopathology. Results are shown graphically in Fig. 4.

The few mice surviving the acute stage of infection represented above developed further symptoms which are illustrated in Fig. 5.

It is possible to divide the course of infection into five phases, viz.:

1. Primary liver invasion.
2. Encephalitis and oily hair effect.
3. Quiescence.
4. Alopecia.
5. Secondary jaundice.

Phase 1 represents the primary invasion of the liver and the establishment of a focus of infection therein. Within 48 hours of an intraperitoneal inoculation the virus can be found in relatively high concentration in the liver and the

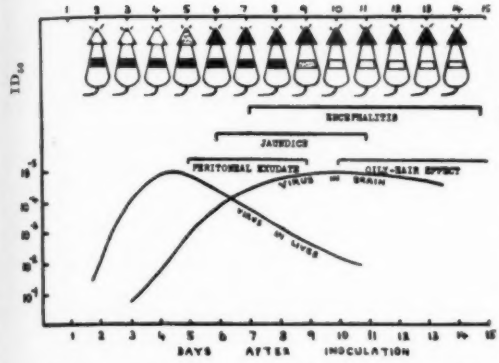


Fig. 4. Probable course of infection in suckling mice during acute stage. Black shading indicates lesions in liver and brain.

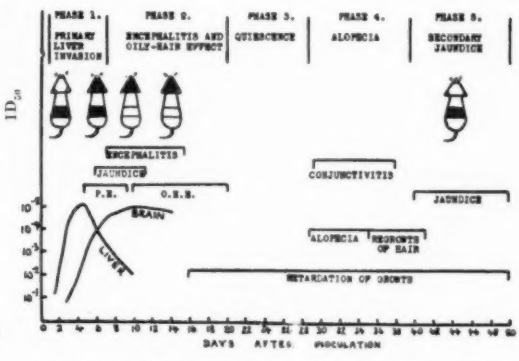


Fig. 5. Probable course of infection in suckling mice covering the entire length of illness. Black shading indicates lesion in liver and brain.

characteristic lesions begin to appear. In phase 2, the predominant features are encephalitis and OHE which may occur independently or together. Some mice may show primary jaundice, especially if a peritoneal exudate is obvious. The virus is found in higher concentration in the central nervous system than elsewhere. The phase of quiescence which is revealed by the few mice surviving the severe primary effects is only apparent from their stunted growth. The phase of alopecia commences with gradual loss of hair on the lower part of the body, the legs and the head. Associated with it may be a condition resembling conjunctivitis. The animals are excitable and the alopecia, of course, produces marked changes in their appearance. With the regrowth of hair may come the final phase of secondary jaundice which lasts almost ten days before the animal dies. This last phase occurs forty to fifty days after inoculation. Little is known, as yet, about the concentration of virus in the tissues of mice at phases 3, 4, or 5, except that the liver and brain from mice at stage 5 are infective for suckling mice. The nature of the disease process so far described in mice indicates that the agent could appropriately be designated an hepato-encephalomyelitis virus.

Following differing routes of inoculation. In a separate experiment, involving 370 mice, infected lung, liver or brain suspensions (diluted from 10^{-1} to 10^{-5}) were inoculated by the intrahepatic, intracerebral or intraperitoneal route into litters of suckling mice. These tests showed (1) the relative frequency of occurrence of the different symptoms, and (2) the concentration of virus in the three tissues following inoculation by the three different routes. The source of virus was seven 9-day old mice showing OHE, peritoneal exudate and jaundice. Separate pools were made of the brain, liver and lungs. The values for the ID_{50} of infected tissues are given in Table 1.

TABLE 1.

Concentration of virus in the tissues of suckling mice following administration of virus by different routes.

Tissue	Negative log. of ID_{50} following inoculation by Intrahepatic route	Intraperitoneal route	Intracerebral route
Lung	4·5	3·9	4·1
Liver	4·1	4·1	4·7
Brain	>4·0	5·5	>5·0

The relative frequency of occurrence has also been recorded in tabular form (Table 2), and indicates the variability of response which occurs irrespective of the route of inoculation.

TABLE 2.

Symptons	Percentage of litters affected
*OHE + Jaundice + Encephalitis	25
OHE + Jaundice	20
Jaundice + Encephalitis	15
OHE + Encephalitis	10
Jaundice alone	10
Encephalitis alone	20
OHE alone	5
Jaundice (Total number)	70
Encephalitis (Total number)	70
OHE (Total number)	60

*OHE = Oily Hair Effect.

Histology of the lesions in suckling mice.

The central nervous system. As a routine, suckling mice were fixed intact in formol-saline and then cut longitudinally so that the brain, the entire length of the cord and such organs as the lung, heart, liver, pancreas and kidney could be seen in one section. Sections were stained with haematoxylin-eosin. A meningitis was occasionally observed. Haemorrhages, perivascular cuffing and pyknosis were marked throughout the brains and the cords of many infected mice (see Fig. 6).

The liver. There were two types of lesion seen in the liver. The areas of necrosis seen macroscopically were sharply demarcated from the surrounding liver tissue (see Fig. 7). Whether necrotic areas were present or not, the most consistent and striking lesions in the liver resulted from the accumulation of leucocytes (polymorphonuclear and mononuclear cells) around the bile ducts (see Fig. 8). The lumen of the ducts appeared to be so constricted as to be barely visible in some cases.

Haematological examination. Haematological examination of mice carried out at the later stages of infection showed no significant alteration in the total erythrocyte count, or in the haemoglobin value. There was, however, a significant neutrophil leucocytosis observed in mice with OHE, jaundice or encephalitis.

Fig. 6. Section stained with haematoxylin-eosin showing lesions in the C.N.S. of a mouse with OHE and ataxia. Objective 20×, Ocular 10×, magnification 400×.

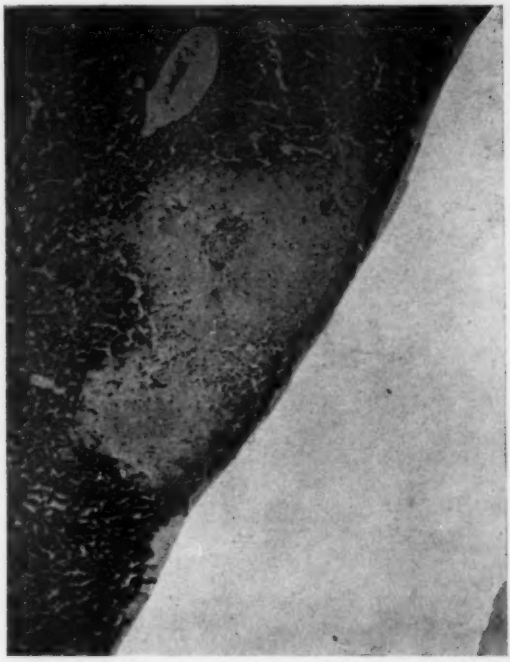


Fig. 7. Section of liver of suckling mouse with OHE. The necrotic areas were in most cases seen macroscopically and were hard and yellow in appearance. Objective 7×, Ocular 5×, magnification 117×.

Properties of the Infective Agent.

The agent is presumed to be a virus because it is filterable through bacteria-tight filters, resistant to the actions of penicillin, streptomycin and aureomycin, and because cultures for bacteria from tissue known to contain the infective agent have been uniformly sterile.

The virus survives heating at 56° C. for two hours, 60° C. for thirty minutes and exposure to 2 p.c. lysol, 0·25 p.c. formalin and 0·25 p.c. phenol at room temperature for one hour. The virus is unaffected by shaking with two volumes of ether and is not inactivated after 1 hour's exposure to phosphate-citrate buffer over a pH range of 2·2 to 8·0. The germicide-resistance

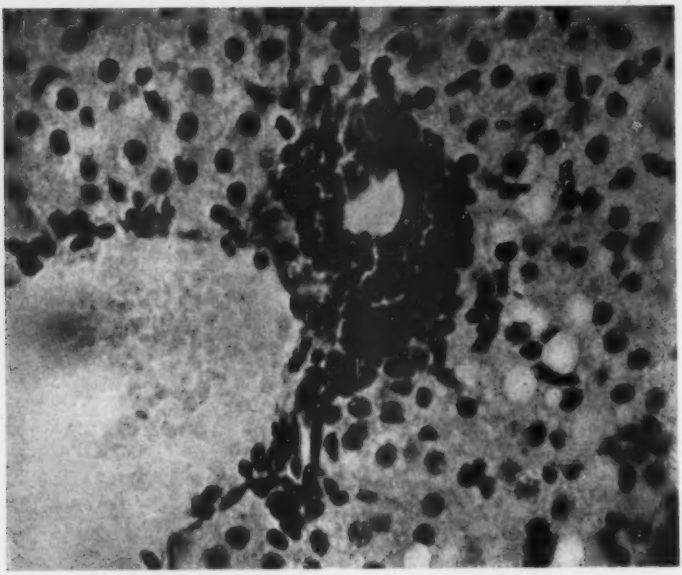


Fig. 8. Section of liver from suckling mouse with OHE shows accumulation of leucocytes around a bile duct. Objective 90×, Ocular 5×, magnification 2,000×.

tests were carried out by the method described by Stanley *et al.* (1951). The heat sensitivity tests were carried out with 10 p.c. infected brain suspensions in broth previously cleared by centrifugation.

Transmissibility.

Infection of laboratory animals other than suckling mice. The only animals apart from suckling mice to show evidence of infection were suckling rats. Animals in which infection could not be demonstrated were guinea-pigs (virus given *per os,* intramuscularly, subcutaneously and by the intraperitoneal route), rabbits (subcutaneously, intravenously and by corneal scarification), monkeys (intracerebral or subcutaneous inoculation) and adult mice (*per os,* intraperitoneal or intracerebral inoculation).

Chorio-allantoic inoculation. The chorio-allantois of 12-day chick embryos was inoculated with infected mouse brain and liver. After 48 hours' incubation at 35° C. discrete white foci were noticed on these membranes. Histopathological examination revealed focal proliferation of ectodermal cells with a small central area of necrosis and eosinophilic accumulation. Inclusion bodies were not demonstrated.

On continued passage, the number of foci produced decreased progressively and none was detectable at the fourth passage. However, the line originating

from mouse brain was continued, and virus was demonstrated in tenth passage material by inoculation of suckling mice with a 10 p.c. membrane suspension.

It was thought that a variant of this virus capable of consistently producing foci on the chorio-allantois might be produced by alternate egg-mouse passage. Pocks were produced by inoculation with mouse brain during each of twelve such passages. (The ID_{50} of a 10 p.c. membrane suspension reached 10^{-4} in the later passages.) However, the foci could not be maintained by reverting to egg-egg passage. Infection did not cause death of the embryo.

Amniotic inoculation. Ten-day old chick embryos were inoculated amniotically with infected mouse brain. After incubating the eggs for three days at 35° C., a 10 p.c. suspension of the chick embryo and membranes, excluding the yolk sac, was used for passage. Virus was detectable by suckling mice inoculation in the first and fifth but not in the twelfth amniotic passage.

The preparation of complement-fixing antigens.

Attempts were made to prepare complement-fixing antigens from the livers and brains of infected suckling mice and from infected chorio-allantoic membranes. The liver preparation proved to be unsatisfactory. Anticomplementary substances were removed from the saline-brain suspensions by alternate freezing and thawing (six times). This was followed by centrifugation at 30,000 r.p.m. in the Spinco ultracentrifuge for 30 minutes. The supernatant fluid was used as the antigen and was stored at —23° C. without preservative.

The antigen from egg-passage virus was prepared by grinding 50 infected chorio-allantoic membranes in 10 ml. saline. The suspension was frozen and thawed six times and cleared by centrifugation. It was stored at —23° C. without preservative.

Complement-fixation tests with human sera.

In complement-fixation tests with human sera the antigen from the infected chorio-allantoic membranes was not used, as it was shown that normal chorio-allantoic membrane and the sera of patients with infective hepatitis fixed complement to a titre of 1/80. Using the complement-fixing antigen prepared from suckling mouse brain, tests were carried out with the sera collected from:

(1) the aboriginal patient from whom the virus was isolated, (2) aboriginal natives at Katherine, Northern Territory (a pool of 10 sera)[1], (3) aboriginal natives at Yuendumu, Northern Territory (a pool of 18 sera)[2], (4) seventeen patients with infective hepatitis, (5) two patients with homologous serum jaundice, (6) six patients with aseptic meningitis, (7) one patient with leptospirosis, and (8) three of the laboratory staff handling tissues infected with the hepato-encephalomyelitis virus.

1 and 2 The authors are grateful to Dr. R. J. Walsh, Director of the Red Cross Blood Transfusion Service, for these pooled sera.

The only human sera which fixed complement specifically were obtained from:

(1) the aboriginal patient from whom the virus was isolated (this serum was collected eleven months after the isolation of the virus from the faeces, and contained complement-fixing antibodies to a titre of 1/20), (2) the aboriginal natives at Katherine whose pooled serum fixed complement to a titre of 1/20 (this pool showed anticomplementary activity to a titre of 1/5), and (3) the aboriginal natives at Yuendumu whose pooled serum fixed complement to a titre of 1/80 (this pool showed anticomplementary activity to a titre of 1/5).

Complement-fixing antibodies to normal suckling mouse brain suspension could not be detected in any of the sera tested.

Attempts to demonstrate the presence of two infective agents.

Because of the variety of symptoms observed it was decided to investigate the possibility of these effects being due to a mixture of two agents. Although the tests have so far indicated that a single entity is involved, it is obvious that the possibility of two viruses being present has not yet been finally excluded. Gledhill and Andrewes (1951) recently demonstrated that a mouse hepatitis virus could be resolved into two filterable components (Gledhill *et al.*, 1952).

TABLE 3.

Complement-fixation tests with hepato-encephalomyelitis virus.

ANTIGEN	Antiserum against A							Antiserum against B						
	RECIPROCAL OF SERUM DILUTIONS							RECIPROCAL OF SERUM DILUTIONS						
	10	20	40	80	160	320	640	10	20	40	80	160	320	640
A	■	■	■	■	◧	◪	□	■	■	■	■	■	◪	□
B	■	■	■	■	■	■	□	■	■	■	■	■	■	□
C	■	■	■	■	◪	□	□	■	■	■	■	◧	□	□
D	■	■	■	■	■	◪	□	■	■	■	■	■	◪	□
Normal Mouse Brain	◪	□	□	□	□	□	□	□	□	□	□	□	□	□
Saline	□	□	□	□	□	□	□	□	□	□	□	□	□	□

A = suckling mouse brains from OHE mice
B = suckling mouse brains from encephalitic mice (via eggs)
C = membrane antigen from infected eggs
D = suckling mouse brains from mice infected with Passage X egg membranes

■ = fixation
□ = lysis

To investigate the possibility of a hepatitis virus and an encephalomyelitis virus being present together, mice were selected showing either the OHE without symptoms of encephalitis or symptoms of encephalitis without the OHE, and complement-fixing antigens were prepared from:

A. brains from suckling mice, showing the OHE, B. brains from suckling mice and suckling rats with symptoms of encephalitis following inoculation with egg-mouse passage material, C. infected chorio-allantoic membranes, and D. brains from suckling mice, infected with membranes following ten successive passages on the chorio-allantois.

Hyper-immune mouse sera were prepared against virus obtained from sources A and B. Fixation of complement was allowed to occur overnight at 4° C. The results are recorded in Table 3.

Not shown in the Table are the control tests for the antigens in saline and normal mouse serum. These were uniformly negative. It will be noticed that the results with the four antigens and the two hyperimmune mouse sera are almost identical. It is felt that this is strong evidence against the possibility of the effects described being caused by a mixture of two infective agents.

DISCUSSION.

The isolation of this hitherto undescribed virus immediately raises the problem of its source—is it a human pathogen, or is it a natural pathogen of rodents? Although this problem cannot yet be adequately answered, some evidence favours the concept that the agent is of human origin. This is suggested by:

(1) the repeated demonstration of the agent in the one specimen of faeces of the patient while hospitalized with a pneumonia, (2) the failure to demonstrate over a period of many years a similar agent in the mouse stock, (3) the presence of complement-fixing antibodies in (a) the patient's serum and (b) pooled sera from aboriginal natives and (4) the absence of antibodies in normal mouse serum.

Complement-fixing antibodies were not demonstrated in the sera of (1) seventeen patients with infective hepatitis, (2) two patients with homologous serum jaundice, (3) one patient with leptospirosis, (4) six patients with aseptic meningitis, and (5) three members of the laboratory staff.

Another hypothesis would be to assume that the particular sample of faeces involved contained a substance or substances capable of stimulating a virus latent in the mouse stock, but there is little evidence to support this.

Until our observations are extended it is difficult to estimate the significance of the finding of complement-fixing antibodies in the sera of aboriginal

natives but not in the sera of white people. On the other hand, the great differences between the white and the black populations of Australia in mode of living and hygiene may well result in qualitative and quantitative differences in antibody to both bacterial and virus diseases. It is worthy of note that two symptoms (alopecia and conjunctivitis) manifest in mice also characterized the illness of the child. The time of onset of alopecia was in each case noticed a considerable time after onset of the disease—32 days for mice and 10 to 11 months for the child. Irrespective of whether the agent is shown to be a natural pathogen of rodents, of man, or of both, the investigations described indicate that the nature of the infection in suckling mice is sufficiently unusual and interesting to warrant further study.

Although the oily hair effect and alopecia appeared at first to be outstanding features of the disease in infant mice, further study soon revealed that the virus itself and the lesions it produced were found primarily in the liver and then in the brain and cord. It is felt that the term "hepato-encephalomyelitis" more accurately describes the properties of the agent than does the term "alopecia virus," and the former designation has been used. Although few data are available on the oily hair effect, its appearance may be connected with a virus invasion of the liver followed by an obstructive jaundice resulting in altered fat metabolism. The OHE appears first at the tail and may be of faecal origin. The fat content of faeces of normal and infected mice has not yet been determined. No skin lesions have been demonstrated. Apart from the observations with the hepato-encephalomyelitis virus, a peritoneal exudate in suckling mice has been observed only following infection with herpes strains. It is interesting to note that both agents cause lesions in the central nervous system of infant mice.

TABLE 4.

	MHV*	HEV†
Liver damage	+	+
Jaundice	+	+
Encephalitis	—	+
Peritoneal exudate	—	+
Oily hair effect	—	+
Alopecia	—	+
Growth on eggs	—	+
Heat stable at 55° C.	—	+

* Mouse Hepatitis Virus. † Hepato-Encephalomyelitis Virus.

The development of a complement-fixation test with the hepato-encephalomyelitis virus has proved satisfactory and, so far, is superior to the suckling-mouse neutralization tests. This finding is contrary to the evidence produced by Stanley and Dorman (1952) for Coxsackie viruses of group A.

An interesting feature is the predilection of the virus for central nervous system tissue. Several passages of brain material given by the intraperitoneal route have resulted in a strain with greater neurotropism, which causes encephalitis in suckling mice after seven days. This tendency to change may be limited by liver passage, but is greatly accelerated by passage on the chorio-allantois.

It is clear that the hepato-encephalomyelitis virus does not share many properties with the mouse hepatitis virus, described by Gledhill and Andrewes (1951). Differences between the two agents are recorded in Table 4. The hepato-encephalomyelitis virus does, however, share some properties with the virus of infective hepatitis in its resistance to heat and germicides.

So far, we have failed to obtain a satisfactory explanation for (1) the appearance of the oily hair effect, and (2) the behaviour of the virus on the chorio-allantois.

There is also insufficient evidence to show whether the virus is a natural pathogen of man or of mice.

SUMMARY.

An account is given of an agent producing infection with unusual symptoms in suckling mice. The agent is considered to be an hepato-encephalomyelitis virus which may cause jaundice, an oily hair effect, encephalomyelitis, retardation of growth, alopecia, conjunctivitis and death in suckling mice. The virus was recovered from the faeces of an aboriginal child with pneumonia who later showed alopecia areata and conjunctivitis. The probable course of infection in mice is outlined and five phases of the disease established. The growth of the virus on the chorio-allantois of chick embryos was demonstrated. Complement-fixation tests with infected mouse brain antigen revealed antibodies in the patient's serum 11 months after isolation of the agent, and in the pooled sera of aboriginal natives from the Northern Territory.

REFERENCES.

Gledhill, A. W. and Andrewes, C. H. (1951): Brit. J. exp. Path., 32, p. 559.
Gledhill, A. W., Dick, G. W. A. and Andrewes, C. H. (1952): Lancet, September 13, p. 509.
Stanley, N. F., Hayes, L. and Dorman, D. C. (1951): Austral. J. exp. Biol., 29, p. 367.
Stanley, N. F. and Dorman, D. C. (1953): *Ibid.*, 31, p. 9.

THE USE OF CHLORAMPHENICOL AND FOSTER MOTHERS IN THE CONTROL OF NATURAL PASTEURELLOSIS IN EXPERIMENTAL MICE

by DAVID F. GRAY AND A. L. CAMPBELL

(From the School of Bacteriology, University of Melbourne).

(Accepted for publication 3rd November, 1952)

The necessity for maintaining experimental animals in complete freedom from natural disease has long been a recognized principle. However, it is becoming increasingly evident that there exists a number of micro-organisms capable of maintaining a delicate host-parasite balance which results in their presence remaining undetected except under special circumstances, such as concurrent infection, continued serial passage of homologous tissue extracts or a lowered plane of nutrition. It can be argued from this knowledge that measures adopted in the past to control naturally occurring diseases in the animal house have been based largely on the false premise that an animal showing no clinical manifestation of infection is, in fact, free of organisms likely to interfere with serological or infection experiments.

In serological work, the incidence of previous immunological stimuli in the form of natural infection, provided the presence of the resulting antibodies is recognized and allowed for, does not present any real difficulties. On the other hand, the presence of potential pathogens which may be completely overlooked or, at best, detected only at the completion of an infection experiment when the animal is sacrificed, may invalidate the experiment or even lead to erroneous conclusions.

With the expansion of our knowledge of chemotherapy, it is not improbable that a time will arrive when adequate precautions may be taken to eliminate most, if not all, potential pathogens from an animal before commencing an infection experiment. Therefore, at the present time it would appear logical to utilize existing facilities to this end as far as is practicable. Whereas the results of actively immunizing laboratory animals have seldom justified the use of this method, there is apparently little or no reason why animals should not be subjected to a chemotherapeutic clearance immediately before use, provided that sufficient interval is allowed for complete elimination of the antibiotic before commencing the experiment, or antibiotics are used which are inactive against the test organism. Experience alone would show whether a chemotherapeutic cover of the latter kind actually during the infection experiment is justifiable.

In any case, the substitution of chemotherapy for the old, wasteful policy of mass slaughter, disinfection and the acquisition of a fresh (presumably healthy) breeding nucleus seems to be applicable in at least some of the common bacterial infections of laboratory animals.

Latent infection of laboratory animals with *Pasteurella pneumotropica* has been described by Jawetz (1950) and Jawetz and Baker (1950). These workers found none of five different breeding establishments surveyed in the eastern United States to be free of this organism and although mice appeared to be the principal host, the organism could also be isolated from guinea-pigs, white rats, cotton rats and hamsters housed on infected premises. From their extensive investigations these workers concluded that the boosting of latent *P. pneumotropica* infection to the clinical level was apparently a function of the intra-nasal passage of mouse lung material or of a sharp decline in host resistance.

Our experience, however, suggests that clinical disease and death from this infection may also occur in breeding animals maintained on a highly nutritive diet and may assume epidemic proportions in a mouse colony.

Attention was drawn to the presence of pasteurellosis in our colony of C57 black mice, in 1949, by the appearance of atypical lesions in the lungs of one or two of a large series of animals inoculated intranasally with pure cultures of *Mycobacterium tuberculosis* H37Rv. (Gray and Mattinson, 1952).

As these mice, which are noted for their high susceptibility to experimental tuberculosis, had been bred here for two years following their importation from America with no evidence of disease, the discovery at autopsy of one or two infected animals did not cause undue concern and the identification of the organism was not taken beyond the generic stage. The appearance of Jawetz's paper, however, led to a detailed examination of the organism which, morphologically, culturally and biochemically was found to correspond to the new species suggested by him. It was concluded, therefore, that the organism which has not been recorded previously in this country was introduced with the mice and remained latent for two years before becoming clinically manifest.

No further cases appeared for several months, but toward the end of 1950, several more animals sacrificed in the course of tuberculosis experiments were found to be suffering from pasteurellosis in the absence of tuberculous lung lesions. Almost a year later, occasional deaths began to occur in breeding animals and *P. pneumotropica* was isolated from purulent lesions involving in some cases almost the whole of the lung tissue in pneumonic changes. At about the same time we encountered serious breeding troubles, including sterility in young does after one litter, a decline in litter numbers and a tendency to eat the young, which were also dying from the disease. Surviving young were unthrifty, a small proportion showed gumming of the eyelids and others shed their hair completely soon after it appeared. At this stage the infection was detected also in albino and multi-coloured strains of mice housed in the same building, becoming evident when these animals were inoculated intranasally with *Haemophilus pertussis*. No spontaneous disease was detected in these strains, however.

The destruction of all breeding units in which cases appeared and rigorous disinfection of premises slowed down, but did not arrest the epidemic, and experimental work on the C57 strain was interrupted. As replacement of the colony presented many difficulties, it was decided to endeavour to eliminate

the infection. Two divergent approaches were attempted, both with apparent success, as no fresh cases have occurred in either breeding or experimental stocks during the past twelve months, nor has the organism since been isolated from normal or diseased lungs. The methods adopted were chemotherapy, the successful use of which is described by Jawetz and Baker (1950), and the fostering of newborn mice on to rats.

EXPERIMENTAL PROCEDURE AND RESULTS.

Isolation of the organism.

The lungs of mice dying naturally or sacrificed in intra-nasal infection experiments were examined for lesions and cultured on 5 p.c. horse blood agar plates aerobically at 37° C. When characteristic smooth, greyish white, non-haemolytic colours appeared, further biochemical and serological examination established their identity. Jawetz (1950) gives a complete description which need not be repeated here.

Chemotherapy.

Jawetz (1950) showed that streptomycin in concentrations of from 2–4 μg/ml. would inhibit broth cultures of *P. pneumotropica* but that penicillin resistance was high; moreover, that addition of streptomycin to infective lung suspensions prior to intranasal instillation effectively controlled transmission of the organism and, finally, that does treated with streptomycin produced offspring free from *Pasteurella.*

As we were infecting the mice with *Myco. tuberculosis* this antibiotic was contra-indicated but a drug sensitivity test, by the routine paper disc method employed in this Department, showed that the organism was sensitive to chloramphenicol.

Administration of chloramphenicol. The extreme bitterness of this antibiotic was considered a possible contra-indication to its addition to the drinking water. However, as this was an easy means of ensuring that a reasonable dose would be consumed by all mice throughout the day it was tried, with success.

Using inverted bottles with a glass nipple, it was found that the average daily consumption was 4 ml. of water per animal (summer). Calculating from the human dose rate of approximately 2 gm. per 70 kg. daily, it was estimated that 20 mice of approximately 20 gm. each would require 11 mg. daily. This figure was well below the toxic dose for mice of 5 mg./20 gm. given by Pratt and Dufrenoy (1949) and it was arbitrarily decided to increase it to 1 mg. per head. The reputed stability of the drug in aqueous solution for at least 28 hours suggested that daily preparation of a bulk sample for all animals would be satisfactory. This was made up by dissolving a 250 mg. capsule in a litre of

water and distributing the solution in bottles to allow for 4 ml. per mouse. The bottles were always emptied within 24 hours, the drug was well tolerated and it appeared that this method of dosing was practicable.

Chemotherapy was continued for two weeks. From the third day of treatment, active hygiene measures were instituted. Groups of mice were transferred to sterilized cages and the mouse room scrubbed, then sprayed with 70 p.c. ethanol. After a further four days this process was repeated, the transfer to sterile cages being completed by the 11th day. After a further three days the treatment was discontinued.

Therapy. Twenty breeding does suffering from advanced clinical pneumonia were isolated and subjected to chloramphenicol therapy at the same dose rate. Representative animals killed at intervals showed rapid bacteriological clearance, but there was little tendency for the extensive lung lesions to resolve even after four weeks' therapy. It is considered, therefore, that sick animals are not likely to respond to chemotherapy in a manner which would justify their retention for breeding or experimental purposes.

The total amount of streptomycin used to treat approximately 2,000 mice for this period was 28 gm.

The use of foster mothers.

Jawetz and Baker have shown, as might be expected in a disease of this nature, that there is no transplacental infection with *P. pneumotropica.* They suggest that airborne infection occurs *via* the upper respiratory tract within the first 48 hours following birth. It was therefore decided to remove the newborn mice from their dam with the minimal possible exposure to organisms excreted from adult respiratory tract.

Apparently healthy pregnant C57 does were isolated and as soon as possible after parturition, the young mice were lifted from the cage with aseptic precautions and transferred to a post-parturient rat whose litter had just been taken away. No difficulty was met in the acceptance of the change and in most cases no losses occurred in the fostered litters.

The fostered mice were maintained in isolation until it was evident that no clinical disease was occurring and the colony from this source has since remained apparently free of the disease.

Had this procedure not proved successful in breaking the transmission chain of the pasteurella, we proposed to remove the litter from the C57 does by caesarian section immediately the first of the litter was born. This exacting, though practicable procedure, however, was not necessary as in none of the exchanges did the organism appear to have been transmitted to the young before the transfer. It is possible, of course, although we did not investigate the point, that the does selected were, in fact, free of infection.

Up till the present time, we have found no evidence of the existence of latent infection in rabbits, guinea-pigs or rats. Other strains of mice housed in close proximity to the C57 colony, however, did become infected and were treated similarly with the same apparently successful result.

SUMMARY.

Pasteurella pneumotropica infection in mice is recorded in Australia. The most probable explanation for its occurrence would appear to be that it existed as a latent infection when C57 black mice were introduced into the country. There was no evidence of its presence in the colony for more than two years.

That this organism, in addition to latent infection, may cause a natural epidemic in adult breeding mice and their litters with sterility, chronic pneumonia and death is shown by our experience.

There is no evidence at present of spread to rabbits or guinea-pigs kept in close proximity to the imported mice, but local mice showed evidence of latent infection after 3 years.

The use of chloramphenicol and of foster mothers to eliminate the infection is described.

REFERENCES.

Gray, D. F. and Mattinson, M. W. (1952): Amer. Rev. Tubere., 65, p. 572.
Jawetz, E. (1950): J. infect. Dis., 86, p. 172.
Jawetz, E. and Baker, W. H. (1950): *Ibid.*, 86, p. 184.
Pratt, R. and Dufrenoy, J. (1949): "Antibiotics" (Lippincott).

POLAROGRAPHIC STUDY OF THE ANTIGEN-ANTIBODY COMPLEX

by B. BREYER AND F. J. RADCLIFF

(From the Section for Clinical Biophysics, The Prince Henry Hospital, Little Bay, N.S.W., and from the Physico-Chemical Laboratory, Faculty of Agriculture, University of Sydney).

(*Accepted for publication 3rd November, 1952.*)

The composition of the antigen-antibody complex has been the subject of numerous investigations (e.g. Pappenheimer *et al.*, 1940; Marrack *et al.*, 1951; Singer and Campbell, 1951; Oncley and Gitlin, 1952; Plescia *et al.*, 1952). Only two physical techniques seem to have been used in the past, viz., the ultracentrifuge and cataphoresis. The disadvantages inherent in these procedures, however, are considerable; they require elaborate apparatus and it is difficult to obtain reliable results. Thus e.g. Marrack *et al.* (1951) write: "The amounts of antigen combined with antibody in the soluble compounds formed in the presence of a great excess of antigen can be studied by electrophoresis or by ultracentrifugation, if the mobility or sedimentation constant of antigen differs sufficiently from that of antibody. Such studies cannot be made by adding excess of antigen directly to an antiserum, or even to a solution of antigen-antibody compound, as the great excess of non-specific proteins would hide the antigen-antibody compound." The authors then proceed to enumerate the methods used to overcome this difficulty. These methods on the whole consist in separating the insoluble antigen-antibody complex, washing and redissolving it in excess antigen, a thoroughly unphysiological procedure.

It is the object of this paper to show that the formation and composition of the antigen-antibody complex can be readily studied polarographically. The advantages of polarography, apart from its simplicity and speed, are that the complex can be studied under near physiological conditions. The method does not rely on the formation of insoluble precipitates and the presence of an excess of non-specific proteins does not interfere.

The use of polarography as a means of investigating the antigen-antibody reaction was the subject of a short note by the present authors (Breyer and Radcliff, 1951). The method follows the observation made by one of us (Breyer, 1939) that the concentration of azoproteins in solution, and therefore their reaction with specific antibodies, can be followed polarographically.

The present communication deals with the details of the technique and with its application to the study of the antigen-antibody complex.

Materials and Methods.

Azoprotein antigen.

Azoprotein was prepared by coupling diazotised p-amino-benzoic acid with egg albumin, following the method of Landsteiner (1932). For polarographic investigation the product obtained from 1 gm. of egg albumin was dissolved in 80 ml. of universal buffer after Prideaux and Ward (1924), pH 7·3. Allowing for an 80 p.c. reaction yield, this gives an approximately 1 p.c. solution of azoprotein.

The azoprotein solution was found to be stable towards light. Even strong sunlight (8 hours) or exposure to ultraviolet light from a mercury lamp (30 minutes) did not appear to decompose it.

The solution was further found stable at room temperature, although it is best kept in the refrigerator to guard against bacterial contamination. A sample was thus kept for four weeks and it was found by daily polarographic as well as viscosity measurements that no deterioration occurred.

To make sure that azoprotein remained unaffected by the presence of mercury in the polarographic cell, a sample was left in contact with mercury for 24 hours. The polarographic step height was unchanged.

Antiserum.

Specific antiserum was prepared by giving rabbits a series of six injections at seven-day intervals. Each injection consisted of 5 ml. of approximately 1 p.c. azoprotein solution in universal buffer, pH 7·3. The rabbits were bled two weeks after the final injection, the blood clotted and the serum separated by centrifugation.

Method.

As several monographs dealing with the polarographic method are in existence (e.g. Kolthoff and Lingane, 1941), experimental details reported in this paper have been restricted to the polarography of azoproteins.

Since dissolved oxygen interferes with the polarographic analysis, solutions must be de-aerated by passing an inert gas (usually hydrogen or nitrogen). Protein-containing solutions, however, tend to retain dissolved oxygen tenaciously and its removal is further made difficult because the rate at which the gas is passed through the sample has to be kept low to avoid undue frothing. It was found best to pass the gas through the cell for a short period in a rapid stream. When the space above the solution was filled with froth, the flow of gas was restricted to one bubble every 1·0–1·5 seconds. Using a microcell (Kolthoff and Lingane, p. 246) and a sample of 5 ml., gassing was continued for at least half an hour. The solution was tested for absence of oxygen by reading the galvanometer deflection at an applied potential of —0·5 volt at intervals of 10 minutes during the de-aeration. The sample was considered ready for analysis when two successive readings differed by not more than 1 p.c.

To obtain reproducible values of potential, a saturated calomel electrode was used as reference half cell in preference to an internal pool electrode.

The characteristic features of an azoprotein polarogram are an appreciably increasing residual current, a gradual start of the step and a steeply increasing diffusion current which shows no indication of becoming constant. The step height, however, can be determined with sufficient accuracy in the usual manner (see Fig. 1).

Additional help can be derived from the fact that beginning and end of the step occur with remarkable constancy at —0·22 and —0·42 volts respectively. All step heights were corrected for residual current by taking a polarogram of serum made up to volume with buffer in proportions corresponding to the azoprotein solution under test.

RESULTS.

Several series of experiments were conducted to test the proportionality of polarographic step height (I_d) and azoprotein concentration. Step heights were measured after adding increasing amounts of azoprotein either to buffer or to non-specific serum. In both cases the proportionality was fairly good (see Fig. 2). The maximum deviations found were ± 5 p.c.

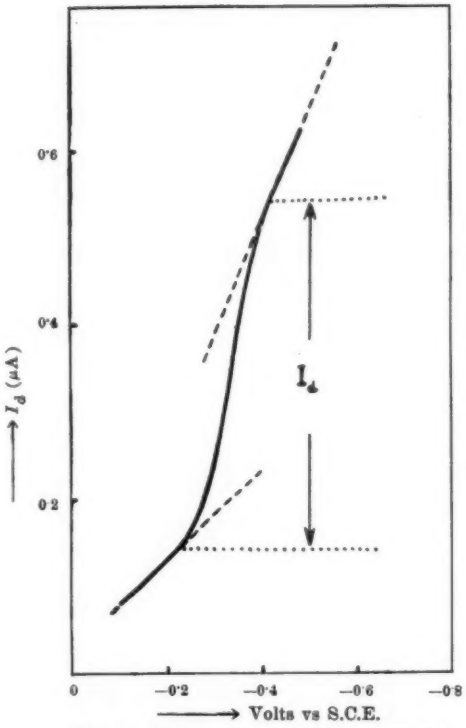


Fig. 1. Polarographic step of azoprotein.

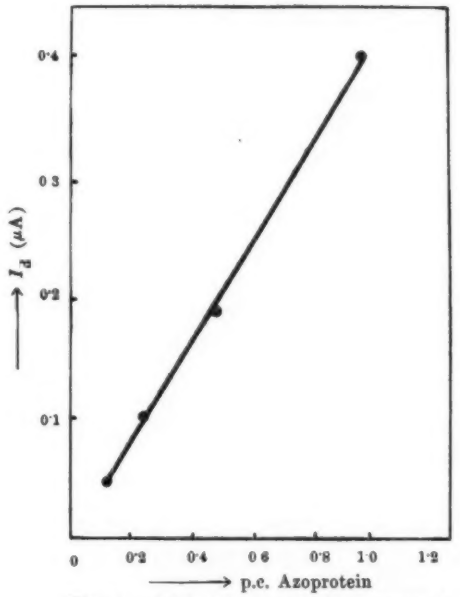


Fig. 2. Calibration curve for azoprotein.

Experiments were then carried out to examine the effect of the addition of specific and of unspecific sera upon the azoprotein polarographic step. Unspecific serum merely acted as normal diluent, whereas the addition of specific antiserum under the same conditions sharply decreased the diffusion current. Table 1 shows the typical results obtained with three out of a considerable number of specific antisera tested.

TABLE 1.

Ratio of unspecific serum to azoprotein solution*	Ratio of antiserum No. 1 to azoprotein solution*	Ratio of antiserum No. 2 to azoprotein solution*	Ratio of antiserum No. 3 to azoprotein solution*	I_d measured μA	I_d† mean value μA	Difference from mean value p.c.
1:1	—	—	—	0·203	0·202	+0·5
—	1:1	—	—	0·152	0·202	−24·8
—	—	1:1	—	0·158	0·202	−21·8
—	—	—	1:1	0·174	0·202	−13·9
2:1	—	—	—	0·139	0·135	+3·0
—	2:1	—	—	0·090	0·135	−33·3
—	—	2:1	—	0·096	0·135	−28·9
—	—	—	2:1	0·109	0·135	−19·3

* All sera were diluted 1:5 with buffer. The azoprotein solution contained approximately 1 p.c. azoprotein.

† As read from the azoprotein calibration curve.

In a further series of experiments the ratio of antigen to antibody was varied by adding increasing amounts of antigen to a given quantity of antiserum. At the same time the total volume of the sample was kept constant by the addition of buffer where necessary. The results obtained are shown in Table 2.

TABLE 2.

Experimental series	Unspecific serum* ml.	Antiserum No. 4* ml.	1 p.c. Azoprotein solution ml.	Universal buffer pH 7·3 ml.	Initial diffusion current (I') μA Individual values†	Initial diffusion current (I') μA Average	Diffusion current at equilibrium (I'') μA Individual values†	Diffusion current at equilibrium (I'') μA Average
a	2·0	—	2·0	—	0·279 0·279	0·279		
	—	2·0	2·0	—			0·211 0·212	0·212
b	2·0	—	1·5	0·5	0·209 0·211	0·210		
	—	2·0	1·5	0·5			0·157 0·153	0·155
c	2·0	—	1·0	1·0	0·141 0·145	0·143		
	—	2·0	1·0	1·0			0·104 0·101	0·103

* All sera were diluted 1:5 with buffer.

† The individual values were obtained from two separate samples prepared by using the same batch of azoprotein and antiserum.

DISCUSSION.

The results reported in the previous section show:

(1) that polarography can be applied to the measurement of azoprotein concentrations, and

(2) that the formation of the antigen-antibody complex is accompanied by a corresponding lowering of the azoprotein step.

It follows that polarography can be used to study the composition of the antigen-antibody complex formed in the presence of an excess of antigen. Thus, if each molecule of antibody (B) combines with x molecules of antigen (A) to give the antigen-antibody complex (C), we can write

$$x\ A + B \rightleftharpoons C$$

$$\text{i.e. } K = \frac{[C]}{[A]^x\ [B]} \quad . \quad . \quad . \quad . \quad . \quad (1)$$

Let the initial concentration of antigen be p moles/litre and that of antibody q moles/litre. If r moles of complex are formed, then at equilibrium

$[C] = r$ moles/litre,
$[A] = p - xr$ moles/litre,
$[B] = q - r$ moles/litre.

For two experimental series i and j, we can write equation (1) in the form

$$K = \frac{r_i}{(p_i - xr_i)^x(q_i - r_i)} = \frac{r_j}{(p_j - xr_j)^x(q_j - r_j)} \quad . \quad . \quad (2)$$

Designating the diffusion current corresponding to the initial azoprotein concentration by I' and that corresponding to the equilibrium concentration by I'', we have

$$p = kI'$$

$$\text{and } p - xr = kI''$$

$$\text{so that } r = k\,\frac{I' - I''}{x}$$

where k is the proportionality constant converting concentrations into currents.

Substituting for r and $p - xr$ in equation (2) and rearranging, we obtain

$$\frac{x\ q_i - k(I_i' - I_i'')}{x\ q_j - k(I_j' - I_j'')} = \frac{I_i' - I_i''}{I_j' - I_j''} \cdot \left(\frac{I_j''}{I_i''}\right)^x \quad . \quad . \quad (3)$$

If $q_j = aq_i$,[1] then from (3)

$$q_i = \frac{K(I_i' - I_i'')[1 - (I_j''/I_i'')^x]}{x[1 - a \cdot (I_i' - I_i'')/(I_j' - I_j'') \cdot (I_j''/I_i'')^x]} \quad . \quad . \quad (4)$$

By substituting for r and $p - xr$ in equation (2) we may also write

$$K = \frac{k(I_i' - I_i'')/x}{k^x I_i''^x [q_i - k(I_i' - I_i'')/x]} \quad . \quad . \quad . \quad . \quad (5)$$

Combining equations (4) and (5) and rearranging, we finally arrive at the expression

$$K.k^x = \frac{(I_j' - I_j'')/I_j''^x - a.(I_i' - I_i'')/I_i''^x}{a\,(I_i' - I_i'') - (I_j' - I_j'')} \quad . \quad . \quad . \quad (6)$$

The results shown in Table 2 were substituted in equation (6) and $K.k^x$ calculated for a series of assumed x values. It can be seen from Table 3 that

[1] In practice the concentration of antiserum was kept constant so that $a = 1$.

the $K.k^x$ values for the three experimental series assume practically identical values for $x = 1{\cdot}2$ (graphical solution yields $x = 1{\cdot}23$). Hence the antigen: antibody ratio in the complex investigated was $1{\cdot}2 : 1$, a result which is in good agreement with those reported in the literature.

TABLE 3.

	$K.k^x \times 10^{-7}$		
x	Combination of series a b	Combination of series a c	Combination of series b c
1·0	0·314	0·270	0·235
1·1	1·89	1·76	1·65
1·2	10·9	10·7	10·5
1·3	60·4	62·2	63·7

SUMMARY.

The composition of the antigen-antibody complex was studied polarographically by using azoprotein as antigen. The results obtained were in good agreement with those reported in the literature.

Acknowledgment. The authors are indebted to Mr. S. Hacobian for checking the manuscript and for drawing the figures reproduced in this paper.

REFERENCES.

Breyer, B. (1939): unpublished.
Breyer, B. and Radcliff, F. J. (1951): Nature, 167, p. 79.
Kolthoff, I. M. and Lingane, J. J. (1941): "Polarography." Interscience Publishers, New York.
Landsteiner, K. and van der Scheer, J. (1932): J. exp. Med., 56, p. 404.
Marrack, J. R., Hoch, H. and Johns, R. G. S. (1951): Brit. J. exp. Path., 32, p. 212.
Oncley, J. L. and Gitlin, D. (1952): J. phys. Chem., 56, p. 85.
Pappenheimer, A. M., Lundgren, H. P. and Williams, J. W. (1940): J. exp. Med., 71, p. 247.
Plescia, O. J., Becker, E. L. and Williams, J. W. (1952): J. Amer. chem. Soc. 74, p. 1,362.
Prideaux, E. B. R. and Ward, A. T. (1924): J. chem. Soc., 125, p. 426.
Singer, S. J. and Campbell, D. H. (1951): J. Amer. chem. Soc., 73, p. 3,543.

A SENSITIVE PHOTO-ELECTRIC COLORIMETER

by HENRY FRANCIS HOLDEN[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 7th November, 1952.)

In biochemical analysis it is often desirable to determine a substance when only a small volume of a weakly coloured derivative is available. To obtain reasonable accuracy this requires a long absorption path and either a very sensitive measuring device or a constant light source of high intensity and a photocell capable of enduring it. Various systems have been proposed but, in general, they tend to be rather complex and costly. The one described below, while not fundamentally new, is relatively inexpensive, simple enough for routine use and can be readily made without very elaborate skill or machinery.

The apparatus is mounted on a small optical bench made from a length of round bright steel rod, in order that when necessary it might be used for other types of analysis using different cells. Any convenient form of rigid mounting which completely excludes stray light may be used. The constant light source is a 6 v. 0·45 a. bicycle head-lamp heated with a 6 v. 11 plate car accumulator. A 0·5 ohm resistor in series with the lamp prolongs its useful life. Small brass plates are soldered to the terminal posts of the accumulator and others to the ends of the wires leading to the lamp. The brass plates are cleaned and the appropriate ones clamped to each other with screws and wing nuts. This helps greatly in keeping the intensity of the light steady. There should be no switch in the light circuit.

The light from the lamp passes successively through a plano-convex lens of 25 mm. focal length to render it parallel, a diaphragm having a central circular hole of 3 mm. diameter, an interference filter to select the suitable wave-band, the absorption cell and a bi-concave lens of 25 mm. focal length. The E.E.L. selenium barrier cell of 25 mm. diameter which receives the light passing through the system is so placed that most of its surface is illuminated. This procedure lessens the effect of local irregularities in the optical system or the surface of the selenium cell. The lenses, which have diameters of about 15 mm. were made with the equipment used for grinding spectacle lenses. For a system of this aperture the results are satisfactory.

The galvanometer is a standard mirror instrument with a sensitivity of 4,000 mm. per micro-ampere at one metre. Its critical damping resistance is 23,000 ohms. The leads from the photo-cell are connected to the variable arms of two wireless potentiometers of 1,000 and 10,000 ohms respectively joined by a 12,000 ohm fixed resistor. The galvanometer is connected to the outer ends of

[1] This work was aided by a grant from the National Health and Medical Research Council.

the two potentiometers and is thus always critically damped. For galvanometers of other characteristics other potentiometers are necessary. The same galvanometer is used for a photo-electric fluorimeter. This reduces the general expense of such equipment.

The determinations are made as with any other single photo-cell direct-reading instrument. The absorption cell is not moved during a series of analyses but is filled, emptied and washed with a capillary pipette. Air bubbles must be watched for and gently dislodged. The perspex cell appears to withstand sufficiently the usual aqueous reagents that have hitherto been placed in it. For special work other cells could be made. It is not dismantled for cleaning. The gasket between the glass end-plates and the perspex body is of "plio-film" rubber sheet 0·003 cm. thick.

If the ratio of the length of the absorption path to the volume of the fluid needed, be regarded as the measure of the sensitivity of a colorimeter and expressed in reciprocal sq. cm., the value for many instruments will lie between 0·2 and 0·4. It is often less for industrial appliances. The sensitivity is increased by the use of special micro-cells but these are usually more suitable for very small volumes of a well-coloured solution than for 2 ml., say, of a very pale liquid for which this colorimeter is designed. With an absorption path of 7·7 cm. and needing only 1·5 ml. solution its figure of sensitivity is about 5 while it is not thereby rendered unduly difficult to use. (Fig. 1.)

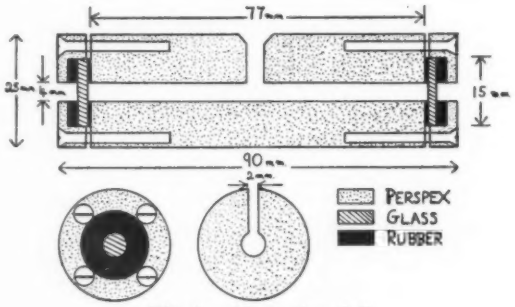


Fig. 1. Absorption Cell.

Analyses for such an instrument include biuret protein determinations on small volumes of sera, analyses of phosphorus or iron or any determination in which drastic economy of material must have precedence over rapidity of execution. The molybdenum blue due to 0·8 microgram of phosphorus in 2 ml. also absorbs about half the incident light. In this Institute the colorimeter is used for the purpose of determining the dye adsorbed by proteins in paper electrophoreses of sera. The paper is cut into transverse strips 0·5 cm. wide and each strip eluted with 2 ml. of dilute alkali. Though in the small test-tubes used the eluates appear almost colourless the ones corresponding to the peaks in the curve of protein distribution absorb over half the light entering the fluid in the absorption cell.

SUMMARY.

A photo-electric colorimeter is described having an optical path through the liquid of 7·7 cm. but requiring only 2 ml. of solution.

THE INTERACTION OF THROMBIN WITH THE PLASMA COMPONENTS OF MAN AND EXPERIMENTAL ANIMALS

by P. FANTL AND L. EBBELS[1]

(From the Baker Medical Research Institute and Clinical Research Unit, Alfred Hospital, Melbourne).

(Accepted for publication 5th November, 1952.)

The addition of thrombin to blood plasma gives rise to several effects. One of them is the formation of fibringel. It is generally assumed that thrombin is the enzyme acting on the substrate fibrinogen. This is supported by experiments of Ferry and Morrison (1947) who found that one part of a thrombin preparation could produce 10^5 parts of fibrin. If this calculation is applied to events in circulating blood it should mean that intravascular liberation of small quantities of thrombin should lead to complete clotting of all the blood in the body. However, such a result can be produced only by drastic experimental procedures. It is therefore obvious that most of the thrombin is consumed in reactions with components other than fibrinogen.

This thrombin-disposing mechanism of plasma appears to be of complex nature. Fibrin can adsorb thrombin as has been recognized by Rettger (1909) and Howell (1910); and Seegers, Nieft and Loomis (1945) have established the quantitative relationship of this adsorption. Finally, Schmidt (1892) observed that thrombin in defibrinated plasma or serum is unstable. Factors termed "antithrombin" are present. The quantitative aspect of this phenomenon has been investigated by several workers including Astrup and Darling (1943), Astrup (1947) and Klein and Seegers (1950). The ability of fibrin to adsorb thrombin and the activity of "antithrombin" are responsible for the disappearance of thrombin in blood.

When thrombin is added to plasma of patients suffering from a variety of illnesses, including obstructive jaundice and multiple myelomata, it is often found that the clotting time is delayed when compared to plasma of normal persons, and a similar phenomenon is observed in the case of certain animal plasmas. The reason for this delay is not known. Since these phenomena have a bearing on problems related to haemorrhage, haemostasis and intravascular coagulation, relevant experiments were carried out and are reported.

[1] This work was carried out with the support of a grant from the National Health and Medical Research Council, Canberra.

MATERIALS AND METHODS.

Blood specimens were obtained by venepuncture from humans, by cardiac puncture from fasting guinea-pigs and rats, and from various blood vessels of rabbits. Ether or nembutal were used as the anaesthetics in experimental animals. One volume of 0·1 M sodium oxalate or one volume of 0·13 M trisodium citrate was mixed with nine volumes of blood. Occasionally amberlite IR100 in the sodium form was used to remove Ca ions.

The plasma specimens were obtained after centrifugation at 5° C. and the cell-free specimens were kept in ice water until the reactions were carried out. These were done at room temperature (20° C.) or in a water bath kept at 37° C. All tests were carried out on the day of blood collection.

The thrombin preparations used were—except for one of bovine origin (Parke-Davis—thrombin topical)—made from various sources following essentially the procedure introduced by Mellanby (1909): To one volume of oxalated plasma, diluted with nine volumes of cold distilled water were added dropwise two volumes of 0·01 M citric acid, and well mixed. The precipitate was collected by centrifugation and the fluid removed by drainage. The residue was dissolved in veronal buffer pH 7·4, half the original plasma volume being used. Solution occurred during warming to 37° C. The addition of one p.c. of 0·1 M $CaCl_2$ produced in one to five minutes a clot which was discarded and the fluid used as thrombin solution. It was generally possible to obtain very active and stable products from human plasma but thrombins from guinea-pig and rat plasma were not always very potent and often deteriorated markedly within half an hour after preparation. Consequently they had to be used immediately after preparation. It has been observed that thrombin can be adsorbed on glass, (Waugh, 1951). But the use of silicone coated apparatus for the preparation of rat thrombin did not improve its stability. Seegers (1952) reported a rapid disappearance of freshly prepared thrombin in dog's plasma which he considers to be due to a thrombin-destroying principle. This, however, does not seem an adequate explanation of the variable stability of our thrombin preparations.

Two different fibrinogen preparations were used. In some experiments fibrinogen was precipitated from diluted plasma by 0·01 M citric acid in the same manner as for the preparation of thrombin but here the deposit was dissolved in veronal buffer, pH 7·4, containing 0·026 M citrate. Fibrinogen was also obtained from oxalated plasma, from which prothrombin was removed by adsorption on barium sulphate, and fibrinogen was isolated by repeated precipitation with ammonium sulphate to 25 p.c. saturation. It was removed by dialysis against 0·12 M sodium chloride containing 0·026 M citrate. The determination of fibrinogen was carried out in 1 ml. specimens diluted 5–10 times with 0·15 M sodium chloride. Fibrin was produced by addition of 0·5 ml. of 0·25 M calcium chloride or by a potent thrombin preparation. The gel was collected on a glass rod and after standing at room temperature for two hours the fluid imbibed in the clot was expressed and the fibrin washed with saline until soluble proteins were removed. The amount of fibrin was estimated by a biuret technique, or by a micro-Kjeldahl procedure.

EXPERIMENTAL.

Addition of thrombin to pathological plasma resulted in clotting times which are recorded in Table 1.

The first nine patients were suffering from obstructive jaundice. Patient H.A.W. was a case of multiple myelomata. The thrombin preparation was standardized by adjusting the clotting time of the normal control plasma to twenty seconds. The results indicate that most of the patients showed a delayed thrombin clotting time.

TABLE 1.

Thrombin clotting time of pathological plasma.

Patient	Time (secs.)	Prothrombin percentage	Fibrinogen mg. p.c.
J.SHI	30	60–100	—
C.A.S.	36	50	—
E.S.L.	30	50	—
C.O.	28	50	320
R.O.	32	50	280
C.A.L.	30	50	380
D.O.	20	100	—
A.L.B.	30	50	310
R.A.T.	35	70	—
H.A.W.	150	30	—
Normal	20	100	160–320

Reaction mixtures consisted of 0·2 ml. oxalated plasma and 0·1 ml. beef thrombin at 37° C.

When thrombin of bovine origin was added to citrated or oxalated plasma of normal persons, rats and guinea-pigs, the clotting times shown in Table 2 were observed:

TABLE 2.

Clotting times of mammalian plasma using bovine thrombin.

Source of plasma	Clotting times (secs.)
Human	14–20
Rat	34–67
Guinea-pig	32–57

Reaction mixtures consisted of 0·1 ml. oxalated plasma, 0·2 ml. veronal buffer pH 7·4 and 0·1 ml. of bovine thrombin dissolved in veronal buffer. T. 37° C.

The results in Table 2 were obtained with five specimens of rat and guinea-pig plasma. In each case the thrombin clotting time was compared with that of human plasma. Different thrombin concentrations within the range given were used.

The difference in reactivity of bovine thrombin with human, rat, and guinea-pig plasma is also apparent from Fig. 1.

The results indicate that plasma from these animals requires a longer time to clot than does human plasma. From the tabulated figures it can be calculated that the animal plasmas require two to four times more bovine thrombin than human plasma to produce a clot in similar time.

The reason for the apparent inhibitory property of the animal plasmas towards bovine thrombin when compared to human plasma is not clear. It might be possible that thrombin is species specific. This assumption was tested by the use of thrombins isolated from human, rat and guinea-pig plasma.

TABLE 3.

Comparative potency of mammalian thrombins on homologous and heterologous plasma.

Source of thrombin	Source of plasma			Human Animal
	Human	Rat	Guinea-pig	
Human 1	4	1·1	—	3·6
Human 2	3	0·85	—	3·5
Human 3	1·45	0·5	—	2·9
Human 4	1·15	0·15	—	7·5
Human 5	0·8	0·1	—	8·0
Human 6	2	—	0·65	3·1
Average	2·06	0·54	—	3·80
Rat 1	1·3	0·35	—	3·7
Rat 2	1·0	0·25		4·0
Rat 3	0·85	0·15		5·6
Rat 4	0·75	0·2		3·7
Rat 5	0·4	0·1		4·0
Average	0·86	0·21	—	4·1
Guinea-pig 1	0·2	—	0·07	2·9

The experimental conditions were similar to those given in Table 2. For the sake of comparison, the results in Table 3 are expressed in thrombin units.[2]

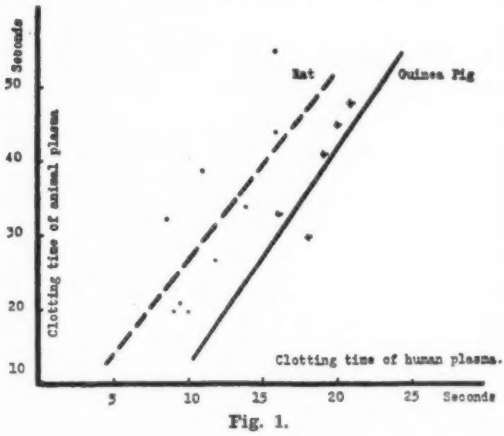


Fig. 1.

From the observed thrombin clotting times it appeared as if all thrombin preparations had greater potency when tested on human plasma than on the animal plasmas. This is indicated by the figures given in the last column of Table 3, which represent the ratio of the thrombin potency in human and animal plasma.

From the tabulated figures it appears that clotting times are dependent upon the potency of the thrombin preparations irrespective of source. Human plasma shows greater reactivity than the animal plasma, similar to the results obtained with bovine thrombin (Table 2).

The results indicate that the thrombin preparations used do not show species specificity.

2 These were calculated from solutions of thrombin preparations, tested under our experimental conditions. A clotting time of fifteen seconds was adopted as one unit. This corresponds very closely to one Iowa unit.

Further experiments were conducted to determine whether the delay in thrombin clotting time shown by the plasma of these animals was due to the presence of an inhibitor of coagulation. For this purpose salmine sulphate was added to the animal plasmas. The thrombin clotting times of such specimens were compared with those of normal human plasma to which heparin was added.

TABLE 4.

Influence of salmine sulphate on thrombin clotting time (secs.).

Addition	Source of plasma			
	Human (270)	Rat (325)	Guinea-pig (315)	Heparinised* human
Nil	17	95	80	115
Salmine** Sulphate	18	88	80	18

Reaction mixtures consisted of 0·4 ml. oxalated plasma, 0·1 ml. veronal buffer pH 7·4 or buffer containing heparin or salmine sulphate and 0·1 ml. bovine thrombin in veronal buffer. T. 37° C. Figures in brackets denote fibrinogen concentration in mg. p.c.

* Conc. of heparin 1·2 γ per ml. of plasma.
** Conc. of salmine sulphate, 10 γ per ml. reaction mixture.

The results indicate quite clearly that the addition of salmine sulphate to the normal animal plasmas did not produce the shortening of the thrombin clotting time to be expected if heparin were present and as indeed took place in the case of heparinized human plasma.

Another reaction to show the presence of thrombin inhibitors in the animal plasma was carried out in mixtures of the latter with human plasma.

TABLE 5.

Thrombin clotting time of heterologous plasma mixtures.

Experiment	1	2	3	4	5	6	7
Plasma	Clotting time in seconds						
Human 100 p.c.	10	10	11	16	10	12	12
50 p.c.	12	14	15	22	14	16	19
Rat 100 p.c.	19	24	39	56	—	—	—
50 p.c.	19	27	36	58	—	—	—
Guinea-pig 100 p.c.	—	—	—	—	24	37	29
50 p.c.	—	—	—	—	36	35	41
Mixtures:							
Human 50 p.c. Animal 50 p.c.	12	16	22	38	13	16	18

Experimental conditions were similar to those in Table 2. The two figures given for the native plasmas represent clotting times for undiluted plasma (100 p.c.) and for specimens diluted 1: 1 with 0·15 M sodium chloride containing 0·02 M sodium oxalate (50 p.c.).

It will be noted from the results given in Table 5 that guinea-pig plasma, despite the fact that it has a delayed coagulation time when compared to human plasma, does not contain an inhibitor of thrombin activity, since mixtures with human plasma show clotting times similar to specimens of human plasma diluted with sodium chloride-oxalate solution. Mixtures of rat and human plasma showed clotting times intermediate between the two results obtained on the original plasmas. In order to obtain more information about the presence of a thrombin inhibitor in rats' plasma, mixtures of native plasma and defibrinated plasma were prepared and their thrombin clotting time determined.

TABLE 6.

Influence of defibrinated plasma on the thrombin clotting time (secs.) of native plasma.

Human plasma (210 mg. p.c. fibrinogen)	Diluent: Sodium chloride, sodium oxalate	Diluent: Human defibr. plasma	Diluent: Rat defibr. plasma	Diluent: G.-pig defibr. plasma
0·4 ml.	14	—	—	—
0·2 ml.	17	18	17	17
0·1 ml.	19	20	20	22
0·05 ml.	27	27	27	28

Reaction mixtures consisted of oxalated plasma or plasma and diluent to give a volume of 0·4 ml., 0·1 ml. veronal buffer pH 7·4 and 0·1 ml. bovine thrombin in veronal buffer. T. 37° C.

The tabulated results indicate that dilution of human plasma with defibrinated plasma of homologous or heterologous origin gave thrombin clotting times identical with dilutions of plasma with 0·12 M sodium chloride and 0·02 M sodium oxalate.

This is taken as further evidence for the absence of thrombin inhibitors in the plasmas. However, the results (Table 6, 7) point to the possibility that the behaviour towards thrombin may be connected with fibrinogen itself.

TABLE 7.

Influence of fibrinogen concentration on thrombin clotting time (secs.).

	Source of plasma: Human (210)	Rat (220)	Guinea-pig (240)
Undiluted	11	39	32
Diluted 1: 1	15	35	32
Diluted 1: 4	34	42	35

Figures in brackets denote mg. p.c. fibrinogen. Experimental conditions were similar to those in Table 2.

Dilutions were carried out with veronal buffer pH 7·4 containing 0·026 M sodium citrate.

It is already known that the thrombin clotting time is dependent upon the fibrinogen concentration of the medium (Deutsch and Gerarde, 1946; Fantl and Nance, 1948). In order to find in what manner the fibrinogens of the different species influence the result, plasmas with similar fibrinogen concentration were selected and are grouped in Table 7.

It will be noted that the effect of thrombin depends very markedly on the fibrinogen concentration, and varies with the species. In the case of human plasma, dilution always resulted in a prolongation of the thrombin clotting times, whilst in the animal plasmas this was not always evident at the dilutions used.

For the following experiments plasma components containing mainly the globulin fraction were prepared by acid precipitation (see Methods). Human and rat preparations are compared with regard to their reactivity with thrombin.

TABLE 8.

Thrombin clotting times (secs.) of rat and human plasma and euglobulin preparations.

Type of preparation	Experiment 1	2	3	4
Human plasma	13 (268)	17 (320)	12 (245)	11 (171)
Human euglobulin	13 (175)	13 (303)	11 (229)	13 (200)
Rat plasma	50 (450)	45 (225)	28 (214)	40 (184)
Rat euglobulin	65 (313)	37 (260)	31 (176)	51 (125)

Figures in brackets denote mg. p.c. fibrinogen. Experimental conditions are similar to those in Table 2. 0·1 ml. of each preparation was taken with 0·2 ml. veronal buffer pH 7·4 and 0·1 ml. human thrombin in veronal buffer. T. 20° C.

The results (Table 8) indicate that no marked differences in the thrombin clotting time between the original plasma and the globulin fraction are noticeable. The absence of "antithrombin" in the globulin fraction was verified in experiments recorded in Table 9.

TABLE 9.

Adsorption of thrombin on fibrin clots.

Source of fibrinogen	Percentage loss of thrombin 15 minutes after coagulation. Expt. 1	Expt. 2	Expt. 3
Human euglobulin	64	55 (175)	70 (200)
Rat euglobulin	56 (190)	40 (314)	20 (125)
Rat fibrinogen	40 (410)	—	20 (282)

Figures in brackets denote mg. p.c. fibrinogen. The reaction mixtures consisted of: 0·2 ml. fibrinogen containing material, 0·4 ml. veronal buffer pH 7·4 and 0·2 ml. human thrombin in veronal buffer. After removal of fibrin 0·1 ml. fluid was tested with 0·2 ml. human, oxalated plasma and the thrombin concentration determined by reference to a curve obtained on the same day. T. 20° C.

Human thrombin preparations which gave a clotting time of 8–11 seconds were used. At varying time intervals after coagulation the remaining thrombin concentration in the fibrin-free fluid was determined.

The thrombin concentration in the defibrinated fluid immediately after coagulation and at time intervals up to 15 minutes later showed no appreciable change. In the majority of experiments the thrombin uptake by human and rat fibrin was of a similar order, and only in a few instances adsorbed rat fibrin less than human material.

Subsequently fibrinogen was further purified by ammonium sulphate precitation and the experiments repeated.

TABLE 10.

Thrombin clotting times (secs.) of plasma and fibrinogen preparations.

Expt. No.	Thrombin source	Human plasma	Rat plasma	Rat fibrinogen	G.-pig plasma	G.-pig fibrinogen
1	Human	15·5 (203)	—	—	35 (390)	11·5 (487)
2	Human	11 (172)	40 (184)	38 (175)	—	—
3	Human	9 (179)	25 (190)	19 (410)	—	—
4	Beef	19 (182)	—	—	41 (465)	20 (472)

Figures in brackets denote mg. p.c. fibrinogen. Experimental conditions were similar to those in Table 2.

From Table 10 it is apparent that fibrinogen isolated from rat's plasma shows no significant difference from the original plasma in the thrombin reactivity. But the fibrinogen separated from guinea-pig's plasma has altered properties. The difference in reactivity compared to human plasma disappeared. It seemed as if the salting out procedure used for the isolation of guinea-pig's fibrinogen had modified the fibrinogen structure.

TABLE 11.

Influence of pyrocatechol on the coagulation time (secs.) of fibrinogen.

Expt. No.	Human plasma	Rat plasma	G.-pig plasma	G.-pig fibrinogen
1	18 (182)	—	30 (465)	24 (362)
1a	13		13	14
2	16 (150)	44 (150)	—	—
2a	12	19		
3	21 (156)	—	28 (300)	—
3a	12		12	
4	12 (406)	—	21 (387)	12 (362)
4a	8		8	8

Figures in brackets denote mg. p.c. fibrinogen.

The composition of the reaction mixtures were similar to those in Table 2. The top lines (1–4) represent results obtained in the absence of pyrocatechol. The lower lines (1a–4a) represent results obtained in the presence of 0·33 p.c. pyrocatechol in the reaction mixture. T. 20° C.

It was of interest to test this assumption. There are a number of substances known to accelerate the thrombin fibrinogen reaction as has been shown by Ferguson (1940), Seegers and Smith (1942) and Gerber and Blanchard (1945). One of the most active of these compounds is pyrocatechol. This was added to the several plasmas as well as to the isolated fibrinogens.

The results (Table 11) show that pyrocatechol-treated guinea-pig plasma behaves like human plasma, but the clotting time of rat's fibrinogen cannot be reduced so strikingly.

DISCUSSION.

Varying activities of thrombin towards animal and bird plasma have been observed by several workers (Quick, 1942), and more recently by Seegers and Smith (1942) and by Burstein (1950).

The experimental results reported in this paper show that thrombin isolated from several mammalian sources—man, ox, rat, guinea-pig—reacts with the plasma of any of these species according to the potency of the thrombin preparation. The origin of the latter is qualitatively of little, if any, significance. From the various plasmas tested, human material was the most active when judged by its reaction rate with any of the thrombins.

In order to explain the delayed thrombin clotting of animal when compared to human plasma, the possibility of the presence of thrombin inhibitors was considered. However, the experiments gave no indication of their presence. On the other hand, it could be shown that the shorter thrombin clotting time of human plasma cannot be due to thrombin activators. In distinction to thrombin inhibitors, whose action is reversible, thrombin inactivating and destroying principles have been recognized in the plasma of all animals.

However, the delay in thrombin clotting time of the animal plasma when compared to human material cannot be due to a difference in "antithrombin" activity, because from the experimental results it appears that native plasma of man and guinea-pig (Table 5), and the defibrinated plasma of man, guinea-pig and rat (Table 6) have similar "antithrombin" activity.

The thrombin clotting time of plasma is of course dependent upon thrombin concentration. In the case of low thrombin potency, the competition between fibrinogen and "antithrombin" for thrombin becomes apparent.

Whilst in the case of human plasma a weak thrombin preparation will still produce a clot containing a maximum yield of fibrin, guinea-pig plasma treated with a similar thrombin preparation gives not only a markedly delayed coagulation time but also a diminished yield of fibrin.

With regard to factors other than "antithrombin" which are responsible for thrombin inactivation in plasma, the adsorption of thrombin by fibrin has been under discussion.

Quick and Favre-Gilly (1949) believe that the voluminous fibrin gel produced in whole blood is capable of disposing of the thrombin quantitatively. Under our experimental conditions approximately 60 p.c. of added thrombin was adsorbed on fibrin. It has to be pointed out that the amount of thrombin present at the time of fibrin gel formation is only a fraction of the potential thrombin amount which can be produced from normal plasma (Smith, 1943), and that the major part of the thrombin is formed after visible clotting has taken place. It appears that the gel once formed does not adsorb thrombin any longer. This is supported by the fact that human serum contains thrombin for several hours after coagulation and in isolated plasma globulin fractions excess thrombin can be detected in the presence of fibrin gel (Table 9).

Inability to demonstrate thrombin inhibitors, taken together with the similar activity of the "antithrombins" of the various plasmas, leads to the conclusion that it is a property of the fibrinogens which is responsible for the behaviour towards thrombin. Several experiments support this assumption. First the fibrinogen present in the euglobulin fraction from rat's plasma shows still a delayed thrombin clotting time similar to that observed in the native plasma. However, on further purification of fibrinogen the thrombin clotting time becomes shorter (Table 10), and, whilst in the case of the rat this is not very marked, isolated guinea-pig's fibrinogen is indistinguishable from human plasma or fibrinogen with regard to the thrombin clotting time. Alteration of the reactivity of fibrinogen can also be demonstrated by the treatment of plasma with phenolic substances, e.g. pyrocatechol. This will reduce the prolonged clotting time of guinea-pig plasma to the level of human material. These facts suggest that the difference in reactivity of thrombin with plasma of different species may be due to differences in chemical and physico-chemical properties of the native fibrinogens. There are already several indications in the literature of species differences in isolated fibrinogens.

Astrup (1950) found that fibrinogens prepared from several species show a difference in the pH optimum of coagulation by thrombin. Ferry and Shulman (1949) noted a difference in the opacity of clots obtained from human and bovine fibrinogen and Morrison *et al.* (1951) observed a difference in the solubility of human and bovine fibrinogen.

In addition to the physico-chemical evidence serologists have repeatedly indicated that fibrinogens are not of identical protein structure in all species (Kenton, 1933).

It is interesting to note that thrombin shows greater affinity for human fibrinogen than it does to animal fibrinogens. This might explain the high incidence of thrombo embolic attacks in humans. On the other hand the delayed thrombin reaction found in the plasma of certain patients could indicate that the liver produces under pathological conditions an abnormal type of fibrinogen.

SUMMARY.

The plasma of many patients suffering from obstructive jaundice and multiple myelomata shows a delayed thrombin clotting time. The plasmas of the rat, guinea-pig and rabbit also show a delayed thrombin clotting time when compared to human material.

The difference in reactivity of thrombin is not due to species specificity of thrombin and is not due to thrombin antagonists.

The phenomenon is explained by a specific configuration of the native fibrinogen, which can be altered in some instances by chemical treatment.

REFERENCES.

Astrup, T. (1947): Act. chem. Scand., 1, p. 744.
Astrup, T. (1950): Advances in Enzymology, Vol. 10, p. 1.
Astrup, T. and Darling, S. (1943): Act. physiol. Scand., 5, p. 13.
Burstein, M. (1950): Compt. rend. Acad. Sci. 230, p. 1,799.
Deutsch, H. F. and Gerarde, H. W. (1946): J. biol. Chem., 166, p. 381.
Fantl, P. and Nance, M. (1948): Med. J. Austral., p. 128.
Ferguson, J. H. (1940): Amer. J. Physiol., 130. p. 759.
Ferry, J. D. and Morrison, P. R. (1947): J. Amer. chem. Soc., 69, p. 388.
Ferry, J. D. and Shulman, S. (1949): *Ibid.*, 71, p. 3,198.
Gerber, C. F. and Blanchard, F. W. (1945): Amer. J. Physiol., 144, p. 447.
Howell, W. H. (1910): *Ibid.*, 26, p. 453.
Kenton, H. B. (1933): J. Immunol., 25, p. 461.
Klein, P. D. and Seegers, W. H. (1950): Blood, 5, p. 742.
Mellanby, J. (1909): J. Physiol., 38, p. 28.
Morrison, P. R., Shulman, S. and Blatt, W. F. (1951): Proc. Soc. exp. Biol. 78, p. 653.
Quick, A. J. (1942): The Hemorrhagic Diseases and the Physiology of Hemostasis, p. 20.
Quick, A. J. and Favre-Gilly (1949): Amer. J. Physiol., 158, p. 387.
Rettger, L. J. (1909): *Ibid.*, 24, p. 406.
Schmidt, Alexander (1892): Zur Blutlehre, Leipzig.
Seegers, W. H. (1952): Arch. Biochem. Biophys., 36, p. 484.
Seegers, W. H., Nieft, M. L. and Loomis, E. C. (1945): Science, 101, p. 520 .
Seegers, W. H. and Smith, H. R. (1942): Amer. J. Physiol., 137, p. 348.
Smith, H. R. (1943): The coagulation of blood: Essays in Biology. Berkeley: Univ. of Calif. Press, p. 549.
Waugh, David (1951): Blood Clotting and Allied Problems: Transactions of the 4th Conference, N.Y., p. 159.

LIBERATION OF N-ACETYL-D-GLUCOSAMINE FROM OVOMUCIN BY MILD ALKALINE HYDROLYSIS

by PETER G. STANLEY[1]

(From the Department of Biochemistry, University of Melbourne).

(Accepted for publication 1st December, 1952.)

Gottschalk and Lind (1949a) showed that ovomucin, a protein fraction from hen eggs, inhibits the haemagglutination of erythrocytes by influenza virus type B, heated at 55° C. for 30 minutes. Compared with ovomucin, ovomucoid, ovalbumin and lysozyme had scarcely any inhibitory activity. Furthermore, it was demonstrated by Fazekas and Gottschalk (1951) that the inhibitory component does not represent more than 5–10 p.c. of the ovomucin fraction. The product of interaction between ovomucin and influenza virus was shown by Gottschalk and Lind (1949b) to be a dialysable, nitrogenous material with considerable reducing power, which after alkaline treatment reacted with p-dimethylaminobenzaldehyde to give a purple colour, identical with that produced by N-acetyl-D-glucosamine in the reaction of Morgan and Elson (1934), but of greater stability. A similar colour reaction with p-dimethylaminobenzaldehyde was produced by dialysates of alkali-treated ovomucin and of all other virus haemagglutination inhibitors known at the time (Gottschalk and Lind, 1949b). Gottschalk and Lind (1949b) considered the virus-split product to be a carbohydrate-peptide complex and on the basis of the Morgan and Elson reaction inferred the presence of an N-acetylhexosamine as part of the carbohydrate moiety. Presence of amino acids in the split product was also demonstrated (Gottschalk and Lind, 1949b). More recent work on the product released from a pure mucoprotein by the viral enzyme has led to a substitution for the original hexosamine concept of one incorporating the "isoglucosamine" structure (Gottschalk, 1951).

Since mild alkaline treatment of both the virus-split product and ovomucin itself produced materials with a positive Morgan-Elson reaction, a more detailed study of the alkaline hydrolysis products of ovomucin was undertaken at the time in the hope that information would be obtained which would throw some light on the nature of the virus-split product. The main findings are presented in this paper.

Materials and Methods.

Ovomucin.

Ovomucin was obtained as a solution in 1 p.c. NaCl by the method of Gottschalk and Lind (1949a). No attempt at further purification was made in this study.

[1] This work was carried out as part of the requirements for the degree of Ph.D. under a Major Research Scholarship awarded by the University of Melbourne.

Ehrlich reagent.

The p-dimethylaminobenzaldehyde component was purified by fractional precipitation from its hydrochloric acid solution with sodium hydroxide. When the reagent was used for N-acetylhexosamine determinations, the procedure of Morgan and Elson (1934) was adopted, using an Eel colorimeter (green filter); for chromatograms the procedure of Partridge (1948) was followed, except that the amount of alkali used in the first spraying reagent was reduced to $\frac{1}{10}$. Acetylacetone, freshly distilled where necessary, was only used when hexosamines were suspected.

Ninhydrin reagent.

This was a 0·1 p.c. (w/v) solution of ninhydrin in n-butanol previously saturated with water.

Paper chromatography.

This was carried out essentially as described by Partridge (1948). Whatman No. 1 and No. 4 papers, prewashed with water, were used for phenol and collidine chromatograms, respectively. Solvents were purified by vacuum distillation. At the conclusion of each run most of the solvent was washed off with anhydrous ether.

EXPERIMENTAL.

Effect of time of heating and NaCl concentration upon yield of alkali-split product from ovomucin.

Thirty ml. quantities of ovomucin in 1 p.c. and 10 p.c. solutions of NaCl were heated for varying times after making 0·1 N with respect to Na_2CO_3. Following adjustment of the salt concentrations to the same value, the reaction products were each dialysed against 100 ml. distilled water at 0° C. and the dialysates then assayed for "N-acetyl-D-glucosamine." The observations are presented in Fig. 1. The results of another experiment to ascertain the effects of varying NaCl concentration with constant time using 30 ml. quantities of ovomucin are set out in Fig. 2. In consequence of these findings, a concentration of 5 p.c. NaCl and a reaction time of 20 minutes were chosen as optimal during the preparation of material for chromatographic studies.

Preparation of dialysates for paper chromatography.

Ovomucin (150–200 ml.), dissolved in 5 p.c. NaCl, was rapidly stirred in a flask immersed in a boiling water bath. 2 N Na_2CO_3 was added to the hot solution to give a concentration of 0·1 N and the reaction allowed to continue for 20 minutes at 96–99° C. To prevent excessive foam formation a few drops of capryl alcohol were added. At the conclusion of the heating, the flask was rapidly cooled in crushed ice and the reaction product, which had lost most of its viscosity, was neutralized to pH 7 with 2 N HCl. During the neutralization some H_2S was evolved. The product was then dialysed against 800 ml. distilled water for 20 hours at 0–1° C. and the dialysate evaporated *in vacuo* to small

volume, the pH being re-adjusted to 7 at intervals during this process. Sodium chloride was removed by a two or three stage process involving precipitation with ethanol to 90 p.c. (v/v) concentration, the supernatants being evaporated to dryness each time. Finally the carbohydrate material in the residue was extracted into dry pyridine according to Malpress and Morrison (1949). Only small losses of material which gave a colour with the Morgan and Elson reagent appeared to occur during the desalting processes. Aliquots of the concentrated and desalted dialysates were then applied to paper in the usual manner and chromatographed with phenol or collidine.

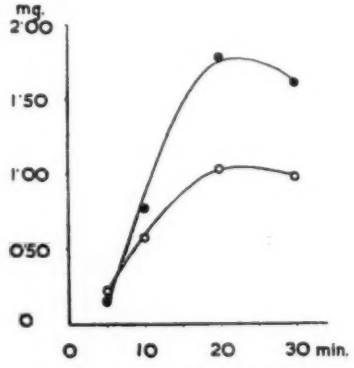


Fig. 1. Yield of N-acetyl-D-glucosamine (mg./100 ml. ovomucin) after hydrolysis with 0·1 N Na_2CO_3 at 100° for varying times in presence of (a) 10 p.c. NaCl (full circles) (b) 1 p.c. NaCl (empty circles).

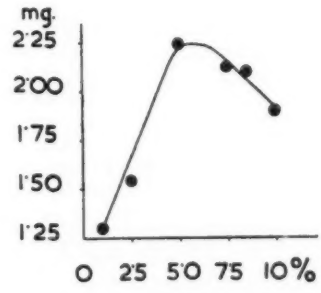


Fig. 2. Yield of N-acetyl-D-glucosamine (mg./100 ml. ovomucin) after hydrolysis for 20 minutes with 0·1 N Na_2CO_3 in presence of varying concentrations of NaCl.

Results of paper chromatography.

The R_F values of substances appearing on the chromatogram after spraying with Ehrlich reagent are set out in Table 1. When the chromatographic runs were made with added authentic N-acetyl-D-glucosamine, no new spots appeared, but there was an intensification of the coloured zone at R_F 0·58. With aniline phthalate (Partridge, 1949) no reducing sugars could be detected; the Ehrlich-reacting substances gave only faint colours with aniline phthalate and none with ninhydrin. Ninhydrin-reacting substances were not consistently observed on the chromatograms and, when present, were only noted in traces.

Elution. Areas of filter paper containing desired substances were located by spraying narrow test strips cut from the chromatograms. For elution the paper was cut into squares about 5 × 5 mm. and covered with large excess of water. Elutions were carried out overnight at 20° C.

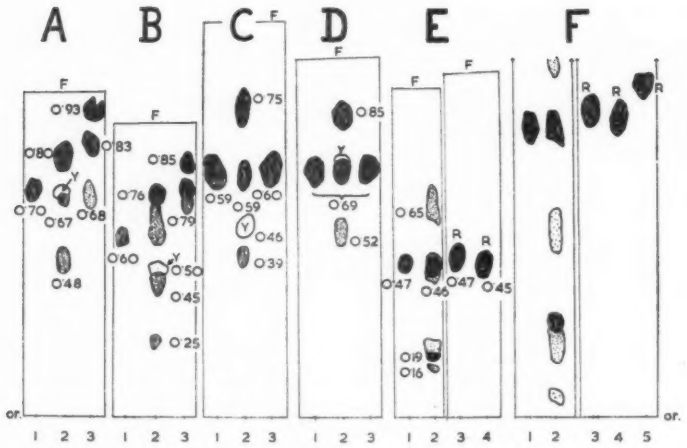


Fig. 3. Tracings of chromatograms.

(A) 1. N-Acetyl-D-glucosamine. 2. Ovomucin hydrolysate, preparation P. 3. Alkali-treated (0·1 N Na_2CO_3) N-acetyl-D-glucosamine (R_F 0·68 spot is very faint). Phenol (NH_3, HCN). Whatman No. 1 paper. Ehrlich reagent.

(B) 1, 2, 3 as in (A). Collidine. Whatman No. 4 paper. Ehrlich reagent.

(C) 1, 3. N-Acetyl-D-glucosamine. 2. Ovomucin hydrolysate, preparation U. Collidine. Whatman No. 4 paper. Ehrlich reagent.

(D) 1, 2, 3 as in (C). Phenol (NH_3, HCN). Whatman No. 1 paper. Ehrlich reagent.

(E) 1, 3. D-Glucosamine-HCl. 2, 4. Acid-hydrolysed ovomucin hydrolysate, preparation P. Collidine. Whatman No. 4 paper. 1, 2. Ninhydrin. 3, 4. Ehrlich reagent (with acetylacetone).

(F) 1, 2, 3, 4 as in (E). 5. N-Acetyl-D-glucosamine. Phenol (NH_3, HCN). Whatman No. 1 paper. 1, 2. Ninhydrin. 3, 4, 5. Ehrlich reagent (with acetylacetone).

Full spots: intense. Dotted spots: faint. All spots are purple except those with R red, Y yellow.

F front, *or* origin of chromatograms.

Acid hydrolysis of eluted materials. The filtered eluate was evaporated to dryness under reduced pressure, the residue hydrolysed at 105° C. for 16 hours with 2 N H_2SO_4 (0·25 ml.). The hydrolysate was diluted with water (4 ml.), 250 mg. $BaCO_3$ added with stirring and, after centrifuging, the excess Ba^{++} was removed from the clear supernatant by 0·04 N H_2SO_4 or by a stream of CO_2. The supernatant was evaporated to dryness under reduced pressure.

Alkali-treatment of N-acetyl-D-glucosamine.

In view of the suggestion (Morgan and Elson, 1934; Aminoff, Morgan and Watkins, 1952) that alkali modifies N-acetyl-D-glucosamine to a cyclic derivative which reacts with p-dimethylaminobenzaldehyde, N-acetyl-D-glucosamine

(100 mg.) was treated with alkali (10 ml., 0·1 N Na_2CO_3) for 15 minutes at 100° C., the solution neutralized with 0·1 N H_2SO_4, the sodium sulphate removed by ethanol precipitation and pyridine extraction. The material proved readily soluble in pyridine in the cold. A control, treated with alkali at 0° C. and then neutralized, was also prepared. Chromatograms of the control showed in each case only a single spot, corresponding to N-acetyl-D-glucosamine (Fig. 3). Material treated with 0·1 N Na_2CO_3 at 100° C. for 15 minutes gave two fast-moving spots of high R_F value (Table 1, Fig. 3). When the experiment was repeated using 0·01 N Na_2CO_3 for 10 minutes, some unchanged N-acetyl-D-glucosamine was present and the faster of the two high R_F spots was less intense or entirely absent.

Chromatograms of N-acetyl-D-glucosamine treated with 0·01 N Na_2CO_3 resemble those of the alkaline hydrolysis product of ovomucin by the presence on each of N-acetyl-D-glucosamine and of one of the fast-moving spots (Table 1 and Fig. 3). There are minor constituents, however, which are present at R_F 0·26 and 0·50 (phenol) and 0·34 (collidine) on chromatograms of the alkaline ovomucin hydrolysate only.

TABLE 1.

Mean R_F values of spots on chromatograms.§

Solvent	D-Glucosamine HCl	N-acetyl-D-glucosamine	N-acetyl-D-glucosamine, 0.1 N Na_2CO_3 (100° C.)	N-acetyl-D-glucosamine, 0.01 N Na_2CO_3 (100° C.)	Product of hydrolysis of ovomucin with 0.05 M Na_2CO_3 (100° C.)	
Collidine*	0·45	0·58	0·76, 0·85	+++ 0·76, ++ 0·58	+ 0·34, ++ 0·58, +++ 0·77	
Phenol**	0·65	0·69	0·83, 0·90	+++ 0·83, ++ 0·69	+ 0·26, + 0·50, ++ 0·69, +++ 0·83, ++ 0·90 ***	

§ For explanation see text. + low intensity. ++ intense. +++ very intense.
* washed Whatman No. 4 paper.
** washed Whatman No. 1 paper, NH_3, HCN atmosphere.
*** present on some chromatograms, fused with R_F 0·83 spot in others.

In order to confirm the presence of N-acetyl-D-glucosamine in the alkaline hydrolysis product of ovomucin, the latter was hydrolysed with 2 N H_2SO_4 in a sealed tube at 105° C. for 16 hours. As controls, N-acetyl-D-glucosamine and alkali-treated N-acetyl-D-glucosamine (0·1 N Na_2CO_3) were treated similarly. D-Glucosamine was demonstrated (Ehrlich and ninhydrin reactions) on chromatograms of the acid-hydrolysed alkaline hydrolysis product of ovomucin (strong +), of acid-hydrolysed alkali-treated N-acetyl-D-glucosamine (trace) and of acid-hydrolysed N-acetyl-D-glucosamine (strong +) (Fig. 3).

When an alkaline hydrolysate of ovomucin was chromatographed with collidine and each of the two main Ehrlich-positive spots eluted and hydrolysed as above, D-glucosamine was recovered from the centre spot (R_F 0·58), thus confirming its identity as N-acetyl-D-glucosamine. Only a trace was detected

in the hydrolysate of the high (R_F 0·78) spot (Fig. 3), probably through overlap of the zones. In addition to D-glucosamine, both chromatograms showed the presence of lesser amounts of ninhydrin-positive materials of low R_F (collidine and phenol).

DISCUSSION.

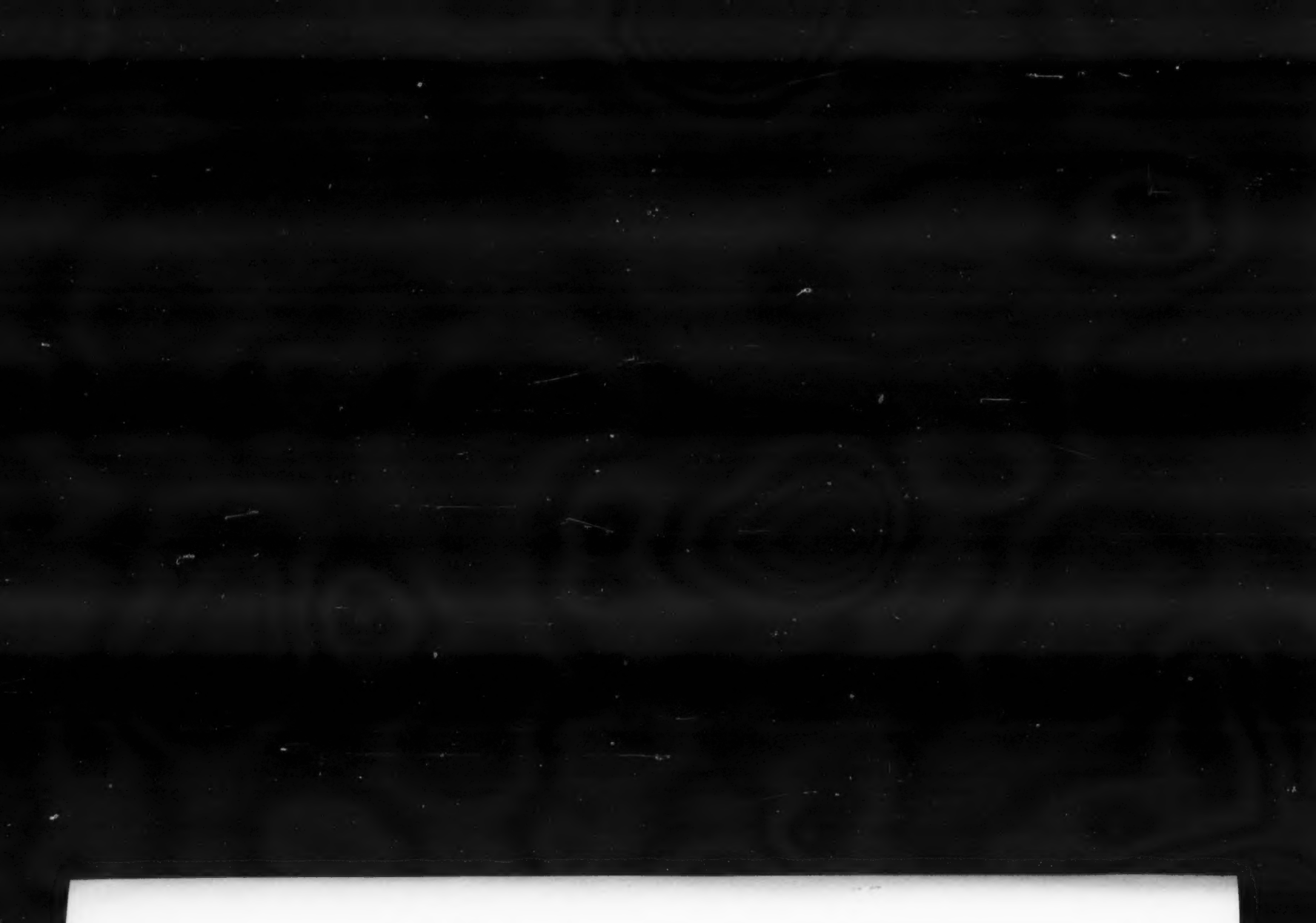
From the evidence presented, it follows that the action of 0·1 N Na_2CO_3 on ovomucin consists primarily of a liberation of N-acetyl-D-glucosamine. In addition there is present a substance apparently identical with one of the products of the action of 0·1 N Na_2CO_3 on N-acetyl-D-glucosamine. The fact that, in this case, the conversion of the N-acetyl-D-glucosamine to high R_F products is virtually complete, whereas some survives the action of alkali on ovomucin, cannot be due to the buffering action of the mucoprotein, as the difference in pH in the two cases was found to be negligible. It can probably be attributed to the gradual release of N-acetyl-D-glucosamine from the mucoprotein, which reaches a maximum after 15 minutes hydrolysis.

It is realized that in the solvents used the R_F values of D-glucosamine and D-chondrosamine are very close (Partridge, 1948; Aminoff and Morgan, 1948) and that for unequivocal identification the use of D-glucosamine and D-chondrosamine as standards would have been desirable. From the results of the chromatograms, however, it seems most likely that the amino sugar is D-glucosamine.

SUMMARY.

Hydrolysis of ovomucin with 0·1 N Na_2CO_3 at 96–99° C. for 20 minutes was found to liberate an N-acyl-hexosamine which appeared to be identical with N-acetyl-D-glucosamine as judged from the results of paper chromatography, both before and after acid hydrolysis. Part of the N-acyl-hexosamine was present as a derivative, similar in chromatographic properties to the main product formed from authentic N-acetyl-D-glucosamine by action of 0·01 N Na_2CO_3.

Acknowledgments. This work was carried out at the suggestion of Dr. A. Gottschalk; the author also wishes to record his thanks to Professor V. M. Trikojus for his interest during this investigation.

REFERENCES.

Aminoff, D. and Morgan, W. T. J. (1948): Nature, 162, p. 579.
Aminoff, D., Morgan, W. T. J. and Watkins, Winifred M. (1952): Biochem. J., 51, p. 379.
Fazekas de St.Groth, S. and Gottschalk, A. (1951): Brit. J. exp. Path., 32, p. 21.
Gottschalk, A. (1951): Nature, 167, p. 845.
Gottschalk, A. and Lind, Patricia E. (1949a): Brit. J. exp. Path., 30, p. 85.
Gottschalk, A. and Lind, Patricia E. (1949b): Nature, 164, p. 232.
Malpress, F. H. and Morrison, A. B. (1949): *Ibid.*, 164, p. 963.
Morgan, W. T. J. and Elson, L. A. (1934): Biochem. J., 28, p. 988.
Partridge, S. M. (1948): *Ibid.*, 42, p. 238.
Partridge, S. M. (1949): Nature, 164, p. 443.